

**Physiological and Pathological Variations
in Urinary Excretion of
Glucose- and Inositol-Containing
Oligosaccharides**

Gudrun Lennartson



Lund 1980

PHYSIOLOGICAL AND PATHOLOGICAL VARIATIONS
IN URINARY EXCRETION OF
GLUCOSE- AND INOSITOL-CONTAINING OLIGOSACCHARIDES

av

Gudrun Lennartson
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PHYSIOLOGICAL AND PATHOLOGICAL VARIATIONS
IN URINARY EXCRETION OF
SULFUR - AND NITROGEN-CONTAINING ORGANIC SUBSTANCES



This dissertation is based on the following communications which will be referred to by the Roman numerals I-V.

- I Quantitation of a urinary tetrasaccharide by gas chromatography and mass spectrometry
G. Lennartson, A. Lundblad, S. Sjöblad, S. Svensson, and P.A. Öckerman
Biomed. Mass Spectrom. 3 (1976) 51-54
- II Glucose-containing oligosaccharides in the urine of patients with glycogen storage disease type II and type III
G. Lennartson, A. Lundblad, J. Lundsten, S. Svensson, and A. Häger
Eur. J. Biochem. 83 (1978) 325-334
- III An α -L-fucopyranosyl-myoinositol in normal human urine
G. Lennartson, A. Lundblad, B. Lindberg, and J. Lönngren
Biochem. Biophys. Res. Commun. 69 (1976) 920-926
- IV On the origin of urinary myoinositol and some oligosaccharides during pregnancy and lactation
G. Lennartson, B.S. Lindberg, A. Lundblad, and T. Löfstrand
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- V Oligosaccharide excretion in urine from humans and the Rhesus monkey
M.A. Chester, G. Lennartson, B.S. Lindberg, and A. Lundblad
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1. INTRODUCTION

There is an increasing interest for the biological role of carbohydrates, particularly on cell surfaces. Different types of cell-cell interactions are mediated by carbohydrates linked either to lipids or proteins in the cell membrane. Cell surface carbohydrates have been shown to be recognition sites for the binding of plasma glycoproteins (1), antibodies (2), lectins (3), toxins (4), enzymes (5), viruses (6), and bacteria (7). There is a need for detailed structural analysis of complex carbohydrates in cell membranes in order to find out more about their physiological function and to be able to moderate or prevent, for instance, the binding of toxins, viruses, and bacteria to the cell. The isolation of glycolipids and glycoproteins from cell surfaces is often a tedious process and only a limited amount of material is usually obtained. An indirect way to obtain structural elements identical to those on the cell surface is to study naturally occurring oligosaccharides and glycopeptides in milk and urine. More than one hundred different carbohydrate compounds have been isolated from these sources and their structures have been established (8,9). Oligosaccharides in urine can also reflect the cellular metabolism of carbohydrates other than cell surface components.

Quantitation of certain compounds during extreme physiological conditions or in various diseases can become a valuable diagnostic tool.

The present investigation will emphasize the methodology

available for quantitation of urinary oligosaccharides, which will be illustrated using two groups of urinary oligosaccharides, namely those containing glucose or myoinositol.

2. QUANTITATION OF OLIGOSACCHARIDES BY GAS-LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Available methods for quantitation of oligosaccharides in biological material have been based on tedious isolation procedures and colorimetric analyses. There was therefore a great need for techniques that would allow a rapid and sensitive determination of individual compounds without previous purification. Gas-liquid chromatography-mass spectrometry (GLC-MS) has proved to be useful for this purpose. Permethylation of reduced oligosaccharides produces volatile derivatives that can be fractionated and quantitated by GLC (10). This technique was adopted for rapid measurement of a glucose-containing tetrasaccharide, (Glc)₄ (I). An oligosaccharide with similar retention time was selected as the internal standard. The permethylated derivatives of both (Glc)₄ and the internal standard, (IM)₄, (Table I) are stable and gave approximately the same response using a flame ionization detector. A glass capillary column was found to give the best resolution, and a linear response was observed over a wide range of injected amounts. Quantitation was accurate to within ±5%. The identification was based on identical retention time and mass spectrum as compared with

TABLE I. Trivial names and structures of the oligosaccharides mentioned in text

Trivial name (abbreviation)	Structure
Glucose tetrasaccharide (Glc) ₄	$\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-Glc}$
Isomaltotetraose (IM) ₄	$\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-6)-Glc}$
IV ₁	$\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-6)-}$ $\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-Glc}$
IV _{2.1}	$\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-Glc}$
IV ₃	$\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-6)-}\alpha\text{-Glc-(1-4)-}\alpha\text{-Glc-(1-4)-Glc}$
Fucosyl- <u>myo</u> inositol	$\alpha\text{-Fuc-(1-0)-myoinositol}$
1- α -Galactinol	$\alpha\text{-Gal-(1-1)-myoinositol}$
Fucosylgalactosyl-inositol (FGIno)	$\beta\text{-Gal-(1-0)-myoinositol}$ $\uparrow (1-2)$ $\alpha\text{-Fuc}$
Difucogalactosyl-inositol (DFGIno)	$\beta\text{-Gal-(1-0)-myoinositol}$ $\uparrow (1-2) \qquad \uparrow (1-0)$ $\alpha\text{-Fuc} \qquad \alpha\text{-Fuc}$
2'fucosyllactose	$\beta\text{-Gal-(1-4)-Glc}$ $\uparrow (1-2)$ $\alpha\text{-Fuc}$
Lactodifucotetraose (LDFT)	$\beta\text{-Gal-(1-4)-Glc}$ $\uparrow (1-2) \quad \uparrow (1-3)$ $\alpha\text{-Fuc} \quad \alpha\text{-Fuc}$
Urine A pentasaccharide	$\alpha\text{-GalNAC-(1-3)-}\beta\text{-Gal-(1-4)-Glc}$ $\uparrow (1-2) \quad \uparrow (1-3)$ $\alpha\text{-Fuc} \quad \alpha\text{-Fuc}$
Urine B trisaccharide	$\alpha\text{-Gal-(1-3)-Gal}$ $\uparrow (1-2)$ $\alpha\text{-Fuc}$

Fucose has the L configuration, all other monosaccharides shown have the D configuration.

those of an authentic compound.

Originally it was considered necessary to make a crude fractionation by gel chromatography (I and III), but later (IV and V) this initial purification was abandoned since direct permethylation of deionized whole urine gave equally good results.

The GLC-MS technique for quantitation of oligosaccharides has been used in several other investigations (11-13). It should be noted that pentasaccharides represent an upper limit in these studies. Hexosamine-containing compounds present special problems and it is often difficult to obtain reproducible response factors.

Oligosaccharides containing charged groups, e.g. sialyl-lactose, can also be quantitated by the above method if special precautions are taken to avoid their removal during the deionizing procedure.

The only available method for rapid quantitation of oligosaccharides larger than pentasaccharides is radioimmunoassay, which was recently used for quantitation of an Le^b -active hexasaccharide in urine of pregnant and lactating women (14). The glucose-containing tetrasaccharide has been determined by a radioimmunoassay using a rabbit antiserum against $(\text{Glc})_4$ coupled to a carrier protein (15). A good correlation was found between the GLC-MS method and the radioimmunoassay.

The advantage of radioimmunoassay is its rapidity when large numbers of samples are to be analyzed.

3. URINARY DEGRADATION PRODUCTS OF GLYCOGEN (I,II,IV)

3.1. Isolation

A number of glucose-containing oligosaccharides that probably represent degradation products of glycogen are excreted under various physiological and pathological conditions.

The general procedure for isolation of these oligosaccharides involved ultrafiltration of the urine and concentration of the ultrafilterable material followed by fractionation by gel chromatography (Sephadex G-25). Glycopeptides and charged oligosaccharides were removed by passage of each gel chromatographic fraction through ion exchange resins of AG-50(H^+) and AG-3(OH^-). Neutral oligosaccharides were separated by descending paper chromatography in different solvent systems until chromatographically homogeneous fractions were obtained.

3.2. Characterization

Structural analysis of purified oligosaccharides was performed using optical rotation, and sugar and methylation analysis. Partial acid hydrolysis of reduced (NaBH_4) oligosaccharides was carried out and the oligosaccharides obtained in the hydrolysate were reduced (NaBD_4) and identified as their permethylated derivatives by GLC-MS. The position of substitution of the pyranose rings in trisaccharides and tetrasaccharides was determined by inspection of the alditol fragments in the b and c series since the intensity ratios

are different depending on the linkage type (II). This method was checked with several known glucose-containing oligosaccharides containing only $\alpha(1-4)$ and $\alpha(1-6)$ linkages. It has not been investigated if this method is applicable to other oligosaccharides. The structures determined using this methodology are given in Table I (IV_1 , $IV_{2.1}$, IV_3).

3.3. Origin

The structures of these oligosaccharides strongly indicate a glycogen origin. The most abundant compound, $(Glc)_4$, has also been isolated by in vitro degradation of glycogen using salivary amylase (16). It can therefore be postulated that $(Glc)_4$ and possibly the larger oligosaccharides (IV_1 , $IV_{2.1}$, $IV_{2.2}$, IV_3) represent 'limit oligosaccharides' obtained by the action of serum amylase on glycogen released from different cells.

As expected the excretion rate for $(Glc)_4$ is considerably increased in patients with pathological accumulation of glycogen (glycogenosis). It has also been demonstrated that patients with Duchenne muscular dystrophy (17) excrete increased amounts of $(Glc)_4$, possibly due to increased leakage of glycogen through defective membranes.

During pregnancy an increased excretion of $(Glc)_4$ is observed, particularly during the 3rd trimester. Soon after delivery the excretion rate returns to non-pregnant values. A high content of glycogen degradation products have been found in a water extract of placenta and might explain the increased urinary excretion. The absence in urine of malto-

dextrins indicates that these molecules are extensively degraded in serum or different tissues.

4. URINARY MYOINOSITOL AND GLYCOSIDES OF MYOINOSITOL

4.1. Free myoinositol

Myoinositol is formed via its 1-phosphate from glucose-6-phosphate (18) and is present in all animal tissues and body fluids investigated (19). The physiological function of the compound, apart from its role in phosphoinositide metabolism, is not fully understood. Myoinositol is freely filtered through the renal glomeruli and in a normal kidney is efficiently reabsorbed by tubular cells (20). The kidney is also the main site of catabolism of free myoinositol (21,22) which is initiated by its oxidation to D-glucuronate (23,24).

Serum (25,26), urine (26), and cerebrospinal fluid (27) of newborn infants have highly elevated amounts of free myoinositol. Adult levels are reached between the 2nd and 5th month of life. Burton and Wells (28) demonstrated that in rats the liver appears to be the main site of myoinositol biosynthesis during fetal life. The high content of myo-inositol in the body fluids of the newborn infant can also in part depend on a dietary supply, since human milk and particularly colostrum is a rich source of myoinositol (29).

In the present investigation free myoinositol was determined in urine of pregnant and lactating women, and in amniotic fluid at various times during pregnancy. The con-

tent of free myoinositol was also determined in placental tissue. The concentration in urine and amniotic fluid showed great individual variation and it is therefore unlikely that myoinositol levels can be used to evaluate the function of the placenta or fetus. A variable dietary intake of free myoinositol is probably the main reason for the great individual variation.

4.2. Fucosyl-myoinositol

1- α -Galactinol was the first glycoside of myoinositol to be described (30). A new glycoside, fucosyl-myoinositol, was isolated from human urine using a similar procedure to that described above (3.1.). Structural analysis revealed an α -L-fucopyranosyl residue linked (1-0) to myoinositol. It has not yet been established which carbon atom in myoinositol is involved in the linkage. The excretion rate of this compound is dependent on ABO blood group secretor status, the highest amounts being found in secretors. The difference in excretion rate between secretors and non-secretors is most pronounced after oral intake of free myoinositol. 1- α -Galactinol, present in the juice of sugar beets, has been shown to be a cofactor in the biosynthesis of certain oligosaccharides acting as a carrier of galactosyl residues. An analogous role for fucosyl-myoinositol is an interesting possibility.

4.3. Pregnancy-dependent glycosides of myoinositol

In a study of human urinary oligosaccharides during

pregnancy and lactation three new myoinositol-containing oligosaccharides were found (31), which appeared to be characteristic of pregnant ABH-secretors and disappeared completely or reached very low levels post partum (11). The structures of these new oligosaccharides are analogous to the urine A-pentasaccharide, lactodifucotetraose, and 2'fucosyllactose, in which the reducing glucose residue in these compounds is replaced by the myoinositol residue. The biological role of these compounds is unknown. In order to obtain information on their origin their excretion rates were compared to that of free myoinositol during both pregnancy and lactation.

It was found that the excretion of free myoinositol is increased during pregnancy and follows the excretion of the glycosides of myoinositol (FGIno, DFGIno). It may therefore be assumed that free myoinositol is a requirement for their formation, but at present it is not known where they are biosynthesized.

Although placenta is a rich source of myoinositol, these oligosaccharides were not detectable in this tissue.

5. THE RHESUS MONKEY - A POSSIBLE ANIMAL MODEL FOR FURTHER STUDIES ON THE METABOLISM OF URINARY OLIGOSACCHARIDES

The biosynthesis of the blood-group-B-trisaccharide has been studied using the Rhesus monkey (32). Since the origin of most urinary oligosaccharides is not known with any

certainly the urine of humans and Rhesus monkeys was compared (V). Many similarities were observed and all the usual human urinary oligosaccharides expected in a blood-group-B-secreter were detected. In addition, the monkeys excreted maltotriose in relatively high amounts, contrary to the human excretion pattern in which this trisaccharide is essentially absent. The presence of maltotriose may indicate a different end point of glycogen degradation in the monkey due to a different glycogen:amylase ratio compared to humans. The products of α -amylase degradation of glycogen are known to depend on enzyme concentration (16).

Monkey urine also contained fucosyl-myoinositol, and in the non-fasting state a large number of inositol-containing oligosaccharides were detected. It is therefore considered that the Rhesus monkey is a useful animal model for the study of the metabolism of glucose- and inositol-containing oligosaccharides under various physiological conditions. This in turn should help to elucidate the mechanisms of oligosaccharide metabolism in humans.

SUMMARY

1. A gas chromatographic-mass spectrometric method for quantitation of a glucose-containing tetrasaccharide has been developed. This method is rapid and sensitive and can be used for quantitation of oligosaccharides not larger than pentasaccharides.
2. Glucose-containing hexa-, hepta- and octasaccharides containing only $\alpha(1-4)$ and $\alpha(1-6)$ linkages have been isolated from urine of patients suffering from Glycogen Storage Disease, types II and III. The characterization procedure involved a new technique to differentiate between internal 1-6 and 1-4 linkages. The oligosaccharide excretion patterns in glycogenosis type II and type III is similar.
3. An α -L-fucopyranosyl-myoinositol has been isolated from urine of ABH-secretors and partly characterized. The compound is also present in small amounts in urine of ABH-nonsecretors. The dependence on secretor status is more pronounced after an oral intake of myoinositol.
4. The excretion of free myoinositol is increased during pregnancy and follows the excretion pattern of some pregnancy-dependent glycosides of myoinositol.

A water extract of placenta has a high content of glycogen degradation products and also a high content of free myoinositol. The site of biosynthesis of the glycosides

of myoinositol occurring in urine of pregnant secretor women is unknown but the placenta could be the major source of a glucose-containing tetrasaccharide excreted in increased amounts during pregnancy.

5. A comparison has been made between the urinary oligosaccharide excretion in humans and the Rhesus monkey. The excretion pattern of oligosaccharides was very similar indicating that the Rhesus monkey should be a useful animal model for study of the metabolism of oligosaccharides.

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Quantitation of a Urinary Tetrasaccharide by Gas Chromatography and Mass Spectrometry†

GUDRUN LENNARTSON, ARNE LUNDBLAD, STURE SJÖBLAD,‡ SIGFRID SVENSSON
and PER ARNE ÖCKERMAN

Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden

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Abstract—A gas chromatographic mass spectrometric method has been developed for the rapid determination of a urinary tetrasaccharide (α -D-Glc-(1 $\rightarrow$ 6)- α -D-Glc-(1 $\rightarrow$ 4)- α -D-Glc-(1 $\rightarrow$ 4)-D-Glc). The urine sample is first fractionated by gel chromatography. An appropriate internal standard is added to the pooled tri-pentasaccharide fraction, which is then reduced, methylated and fractionated by g.c. The identification of the tetrasaccharide derivative is based on the g.c. relative retention time and the mass spectrum of the reduced permethylated tetrasaccharide. The normal excretion rate was in the range of 0.1–2.5 mg per 24 hours. Greatly increased amounts (9.4–89.6 mg 24 h⁻¹) were found in the urine of patients with glycogen storage disease type II and type III and in one patient with unclassified muscular disease. A moderate increase (3.6–8.6 mg 24 h⁻¹) was observed in one patient with glycogen storage disease type VI, in two patients with Duchenne muscular dystrophy and in two other patients with unclassified muscular disease.

Introduction

NORMAL human urine contains a large number of different oligosaccharides, some of which are related to the ABO- and secretor status of the individual.¹ One of the oligosaccharides that does not seem to be related to any known genetic system is a glucose-containing tetrasaccharide: α -D-Glc-(1 $\rightarrow$ 6)- α -D-Glc-(1 $\rightarrow$ 4)- α -D-Glc-(1 $\rightarrow$ 4)-D-Glc [(Glc)₄].² This oligosaccharide was found in small amounts (less than 1 mg 24 h⁻¹) in normal urine and in highly increased amounts (> 13 mg 24 h⁻¹) in the urine of a patient with glycogen storage disease type II (Pompe).² The structure of this compound strongly indicates a glycogen origin and may represent a limit oligosaccharide obtained as a product of amylase action on glycogen released into the circulation.² In order to obtain further support for this hypothesis it was considered valuable to determine the excretion rate of this oligosaccharide in a number of different diseases where increased glycogen degradation might be expected. This prompted us to develop a g.c.m.s. method that would allow a rapid and accurate determination of this oligosaccharide.

Experimental

PATIENTS STUDIED

Studies were performed in a total of ten normal subjects, two patients with glycogen storage disease (GSD) type I,³ one patient with GSD II,² four patients with GSD III, one patient with GSD VI, two patients with Fabry's disease, two patients with GM₁-gangliosidosis type II, one patient with Gaucher's disease and

two patients with Duchenne muscular dystrophy (DMD). For the patients with GSD III, GSD VI, Fabry's disease and GM₁-gangliosidosis type II the diagnosis was made from the clinical picture and the demonstration of the typical enzyme deficiency. The DMD cases were classified on the basis of typical inheritance, clinical signs, serum elevations of creatinine, phosphokinase and lactate dehydrogenase, electron microscopy of muscular tissues, and electromyography. Eight children admitted to hospital because of muscular weakness were also examined. Available data did not allow an exact classification of those patients.

URINE

A 24 h urine sample was collected from each of the individuals studied. Bacterial growth was prevented by the addition of phenylmercuric nitrate (30 ml saturated solution per litre of urine).

OLIGOSACCHARIDES

The glucose-containing tetrasaccharide (Glc)₄ was isolated from normal urine as previously described.² Isomaltotetraose (IM)₄ was a gift from Dr Kirsti Granath, Pharmacia, Uppsala, Sweden.

GEL CHROMATOGRAPHY

A column of Sephadex G-25 fine (2.8 $\times$ 145 cm, void volume 400 ml) was used in all gel chromatographic experiments.

ANALYTICAL METHODS

Total hexose was determined by the anthrone method.⁴ Methylation of reduced oligosaccharides was carried out as described elsewhere.⁵ Permethylated reduced oligosaccharides were separated on a Perkin-Elmer 900 gas chromatograph equipped with a glass capillary column (25 m $\times$ 0.25 mm, wall-coated with

† Abbreviations: GSD = glycogen storage disease; DMD = Duchenne muscular dystrophy; (Glc)₄ = glucose-containing tetrasaccharide α -D-Glc-(1 $\rightarrow$ 6)- α -D-Glc-(1 $\rightarrow$ 4)- α -D-Glc-(1 $\rightarrow$ 4)-D-Glc; (IM)₄ = isomaltotetraose.

‡ To whom correspondence should be addressed.

SE 30). The separations were performed at 340 °C. A Varian MAT 311A g.c.m.s. instrument fitted with the same column was used, together with the Spectroscopy 100 MS data system. Mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 1 mA and an ion source temperature of 120 °C.

Results

DETERMINATION OF THE RESPONSE FACTOR WITH STANDARD COMPOUNDS

Different amounts of purified (Glc)₄ were reduced with sodium borodeuteride. The reduced samples were methylated together with a constant amount (500 µg) of reduced (IM)₄. The permethylated oligosaccharides were fractionated by g.c. and the relative retention times and peak areas were calculated. A linear response was obtained over a wide range (1–700 µg) ($k = 1$). The mass spectrum of the reduced and permethylated (Glc)₄ was recorded (Fig. 1).

DETERMINATION OF (Glc)₄ IN DIFFERENT URINE SAMPLES

One tenth of the total 24 h urine sample, concentrated to 10 ml by rotatory evaporation was fractionated on a Sephadex G-25 column. A typical elution pattern is

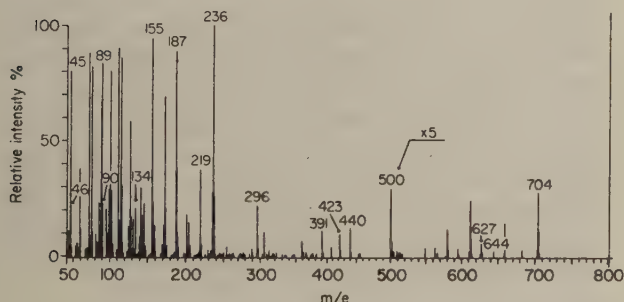


FIG. 1. Mass spectrum of reduced and permethylated (Glc)₄.

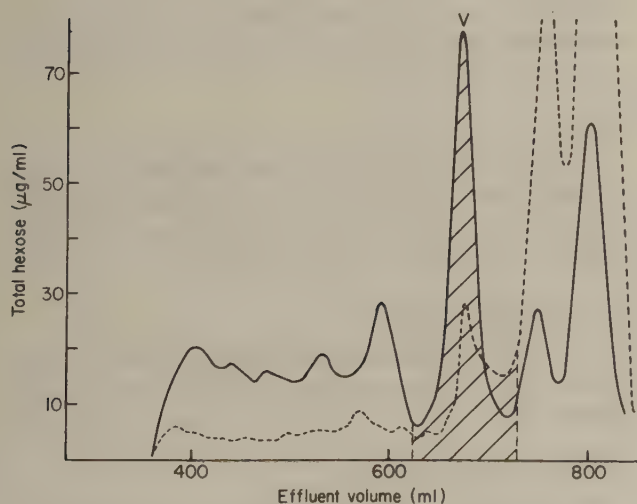


FIG. 2. Gel chromatographic distribution (Sephadex G-25 fine) of hexose-containing material in urine of a normal individual (---) and a patient with GSD III (M.J.) (—).

given in Fig. 2. Fractions corresponding to the tri-pentasaccharide region were pooled as indicated in Fig. 2 and concentrated to dryness. 500 µg of (IM)₄ were added as internal standard and the sample was reduced with sodium borodeuteride, dissolved in dimethyl sulphoxide and methylated. The permethylated urinary oligosaccharides were analysed by g.c.m.s. as outlined above for the standard compounds. A representative gas chromatogram is given in Fig. 3. The mass spectrum for the compound with the same retention time as authentic (Glc)₄ was recorded, and compared with that of the standard compound. The spectra were identical. In Table 1 the excretion rate of (Glc)₄ in 10 normals and 23 patients is given. Normal excretion rate was in the range of 0.1–2.5 mg 24 h⁻¹. The highest excretion (89.6 mg 24 h⁻¹) was observed in one patient with GSD II (Pompe). Four patients with GSD III and one patient

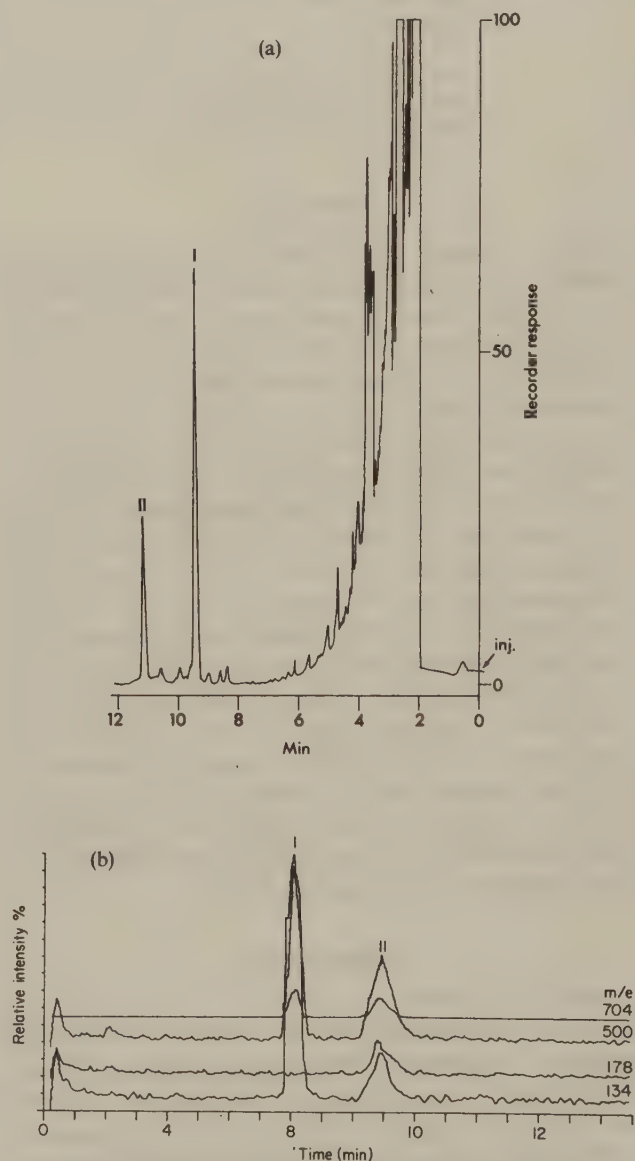


FIG. 3. Gas liquid chromatographic analysis of (Glc)₄ from a patient with GDS III (L.W.). Peak I represents (Glc)₄ and peak II the internal standard (IM)₄. (a) Flame ionization detector; (b) multiple ion detection.

TABLE 1. Excretion rate of (Glc)₄ in 10 normals and 23 patients with different diagnoses

Subject	Age (years)	Diagnosis	mg (Glc) ₄ 24 h ⁻¹ urine
Controls (n = 10)	5-20	—	0.1-2.5
K.T.	24	Glycogenosis type I	0.2
S.T.	16	Glycogenosis type I	2.3
T.L.	12	Glycogenosis type II	89.6
J.W.	7	Glycogenosis type III	16.7
L.W.	10	Glycogenosis type III	12.8
M.J.	4	Glycogenosis type III	45.2
T.J.	6	Glycogenosis type III	9.4
P.A.	10	Glycogenosis type VI	5.0
A.G.	25	Fabry's disease	0.5
B.S.	42	Fabry's disease	0.6
M.A.	11	GM ₁ -Gangliosidosis type II	0.1
U.A.	10	GM ₁ -Gangliosidosis type II	0.7
J.H.	9	Gaucher's disease	0.3
M.A.	8	Duchenne's muscular dystrophy	4.4
B.A.	13	Duchenne's muscular dystrophy	8.6
M.S.	15	Unclassified muscular disease	2.0
T.M.	4	Unclassified muscular disease	13.3
M.B.	2	Unclassified muscular disease	1.4
A.T.	5	Unclassified muscular disease	1.5
A.F.	3	Unclassified muscular disease	7.0
F.K.	4	Unclassified muscular disease	0.6
P.M.	6	Unclassified muscular disease	2.3
B.J.	27	Unclassified muscular disease	3.6

with unclassified muscular disease also had a considerably increased excretion (9.4–45.2 mg 24 h⁻¹). A moderately increased excretion (3.6–8.6 mg 24 h⁻¹) was observed in one patient with GSD VI, two patients with DMD and two patients with unclassified muscular disease. The other patients studied fell in the normal range.

Discussion

The identification and quantitation of (Glc)₄ from the urine of different individuals was performed by g.c.m.s. In order to facilitate the g.c. separation of the oligosaccharide mixture obtained in the tri-pentasaccharide region, the oligosaccharides were reduced into their corresponding alditols before methylation. This procedure eliminates the formation of a mixture of anomeric derivatives. The methylated oligosaccharide alditols were found to be ideal for g.c. Thus, both oligosaccharides studied were stable and gave approximately the same response by flame ionization detector. In order to get good resolution in the separation, a glass capillary column was used. Furthermore a linear response was observed with a wide range of injected amounts.

The region of the gas chromatogram in which reduced and permethylated (Glc)₄ appeared was relatively free of other components, making the quantitation of (Glc)₄ reliable within $\pm 5\%$. The urinary blood group active A- and B-trisaccharides⁷ and the trisaccharide

characteristic of mannosidosis patients,⁸ which elute in region V of the gel chromatogram, appear earlier in the gas chromatogram and thus do not interfere with the analysis of (Glc)₄.

The fragmentation pattern of reduced and permethylated authentic (Glc)₄ is given in Fig. 4. The A-series of fragments⁶ shows aA₁ (m/e 219), aA₂ (m/e 187), aA₃ (m/e 155), baA₁ (m/e 423) baA₂ (m/e 391) and cbaA₁ (m/e 627). These fragments are obtained from three hexose units (a, b, c) linked together. The J-series of fragments are AldJ₁ (m/e 296), bcAldJ₁ (m/e 500) and abcAldJ₁ (m/e 704). These fragments, together with the fragments Ald (m/e 236), cAld (m/e 440) and bcAld (m/e 644) and the A-series of fragments, show the linear chain of four hexose units to be present in (Glc)₄. That hexose unit c is linked to the 4 position of hexose unit d is evident from the fragments obtained by cleavage of the alditol chain (Fig. 4). The linkages between the other hexose units cannot be conclusively ascertained from the mass spectrum of the permethylated oligosaccharide alditol until further studies on the fragmentation pattern have been performed. However, the identification of (Glc)₄ in the different urine samples based on the complete identity of both relative retention time and mass spectrum with those from authentic (Glc)₄ samples, must be considered to be fairly certain.

The structure of (Glc)₄ indicates a glycogen origin. The finding of increased amounts of this oligosaccharide in the urine of patients with GSD II, III and VI gives more support for this hypothesis. The two patients with GSD I had an excretion of (Glc)₄ within the normal range. This may indicate that (Glc)₄ excretion is not directly related to glycogen storage. However, those patients had reached a late stage of the disease with mild symptoms and they may not be representative for the disease in earlier stages where storage symptoms are more prominent. The differences in enzymatic defect and structure of stored glycogen in different types of GSD evidently does not affect the structure of this predominant glucose-containing oligosaccharide excreted in the urine of these patients. The finding of increased excretion of (Glc)₄ in two patients with Duchenne muscular dystrophy and in three out of eight patients with unclassified muscular disease, might indicate differences in etiology in the muscular dystrophy group of diseases. The increased excretion in some of them might reflect an abnormal glycogen metabolism.

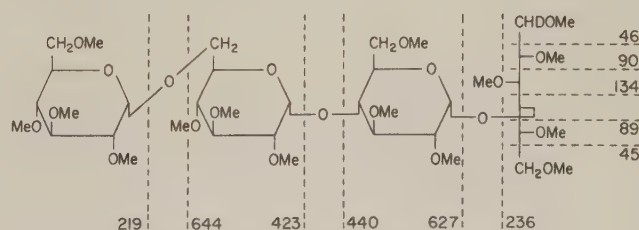


FIG. 4. The most characteristic primary fragmentations of reduced permethylated (Glc)₄.

Our results suggest that this method may be of additional use in identification of certain glycogen storage diseases and muscular dystrophies. However, a larger series of patients will have to be studied before the clinical value of (Glc)₄ determinations can be settled.

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Glucose-Containing Oligosaccharides in the Urine of Patients with Glycogen Storage Disease Type II and Type III

Gudrun LENNARTSON, Arne LUNDBLAD, Jörgen LUNDSTEN, Sigfrid SVENSSON, and Anders HÄGER

Department of Clinical Chemistry, University Hospital, Lund, and Department of Paediatrics, University Hospital, Linköping

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Patients with glycogen storage disease type II and type III were recently found to excrete increased amounts of a glucose-containing tetrasaccharide $\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlc}$ [Lennartson, G., Lundblad, A., Sjöblad, S., Svensson, S. and Öckerman, P. A. (1976) *Biomed. Mass Spectrom.* 3, 51–54]. In addition to this tetrasaccharide, urine from these patients also contains larger oligosaccharides containing only glucose. From urine of patients with glycogen storage disease type II and type III, three and four oligosaccharides respectively have been isolated. Structural studies including sugar analyses, methylation analyses, partial acid hydrolysis and optical rotation revealed that three compounds were present in the urine of both patients. Their proposed structures or partial structures are as follows: $\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlc}$, $\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlc}$, and $\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlc}$. A fourth compound has been partially characterized as a branched heptasaccharide with four (1→4) linkages and two (1→6) linkages.

Glycogen is possibly the origin of these compounds. However, the number of (1→6) linkages is higher than expected and may indicate a shorter distance between branches in glycogen than has been generally assumed.

It was recently reported that a patient with glycogen storage disease type II excreted increased amounts of a glucose-containing tetrasaccharide $(\text{Glc})_4$ [1], the excretion rate of which was determined using a newly developed gas-liquid chromatography/mass spectrometry method [2]. Normal excretion rate is in the range of 0.1–2.5 mg/24 h, but greatly increased amounts were found in the urine of patients with glycogen storage disease type II and type III. Preliminary studies have indicated that the urines of these patients also contain a number of larger glucose-rich oligosaccharides in addition to the main tetrasaccharide [1], but the origin of these oligosaccharides is not known although it is possible that they represent degradation products of accumulated glycogen. The structure of glycogen in glycogen storage disease

type II has been reported to be normal [3], whereas in type III an abnormal glycogen (denoted 'limit dextrin') is accumulated [4]. The aim of this investigation was to determine the structures of the larger oligosaccharides from both glycogenosis type II and type III urine, and to see if any differences could be correlated with known structural differences between the two types of glycogen.

EXPERIMENTAL PROCEDURE

Materials

24-h urine specimens were collected from two healthy boys (aged 10 and 12) and from a ten-year-old boy with the childhood form of glycogen storage disease type II. The clinical description of this patient has been reported [1]. From a four-year-old girl (M. J.) with glycogen storage disease type III, 24-h urine portions were collected. She had an enlarged liver and laboratory investigations showed elevated serum aminotransferases and lactic dehydrogenase,

Abbreviations. Glc, glucose; Me, methyl; $(\text{Glc})_4$, $\text{DGlcp}(\alpha 1 \rightarrow 6)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlcp}(\alpha 1 \rightarrow 4)\text{DGlc}$; abbreviations for other oligosaccharides follow IUPAC/IUB Recommendations, see *Eur. J. Biochem.* 79, 17 (1977), all residues being D-glucopyranose residues and all linkages α ; in fragments, a reducing group with an asterisk, –Glc*, indicates the reducing group residue of the original oligosaccharide.

a low fasting blood sugar, elevated fasting serum lipids and intermittent ketonuria in the morning urine. A liver biopsy revealed an increased glycogen content and a complete absence of amylo-1,6-glucosidase activity. From these data it is evident that the patient can be diagnosed as having glycogen storage disease type III.

Oligosaccharides

Panose was purchased from BDH Chemicals Ltd (Poole, England). The glucose-containing tetrasaccharide (Glc)₄ was isolated from normal urine as previously described [1]. Isomaltotriose and isomaltotetraose were gifts from Dr Kirsti Granath (Pharmacia, Uppsala, Sweden). Maltotetraose was isolated from a partial hydrolysate of starch.

General Methods

Bacterial growth was prevented by the addition of phenylmercuric nitrate (30 ml of saturated solution per l of urine). The urines were filtered and ultrafiltered at 4 °C as previously described [5]. Ultrafiltrates and chromatographic fractions were concentrated by rotatory evaporation (water bath temperature 40 °C). Gel chromatography was carried out using a Sephadex G-25 (fine) column (10 × 108 cm, void volume = 2800 ml) eluted with distilled water containing phenylmercuric nitrate as above and with a flow rate of 6 ml/min, and a Bio Gel P-2 (minus 400 mesh) column (1.5 × 87 cm, eluent = distilled water, flow rate = 0.25 ml/min, void volume = 48 ml). Preparative zone electrophoresis in 2 M acetic acid was carried out using the block method of Kunkel [6] on Pevikon C870 [7]. Whatman no. 3 papers were used for descending paper chromatography in the following systems: (a) propan-1-ol/ethyl acetate/water (6/1/3, v/v/v), (b) ethyl acetate/pyridine/acetic acid/water (5/5/1/3, v/v/v/v), (c) ethyl acetate/pyridine/water (10/4/3, v/v/v), (d) propan-1-ol/ethyl acetate/water (45/35/23, v/v/v), (e) ethyl acetate/acetic acid/water (3/1/1, v/v/v), (f) ethyl acetate/pyridine/water (2/1/2, v/v/v, upper phase). Papers were stained with a silver dip reagent [8].

Analytical Methods

A colorimetric method was used for the determination of total hexose [9]. Sugar analysis was performed by gas-liquid chromatography [10] and mass spectrometry [11]. Methylation analysis was performed as previously described [12]. A Perkin-Elmer 3920 gas-liquid chromatograph was used under the following conditions: (a) glass column, 2 m, packed with 3% ECNSS-M w/w on Chromosorb Q (100–200 mesh) at a column temperature of 185–210 °C for sugar

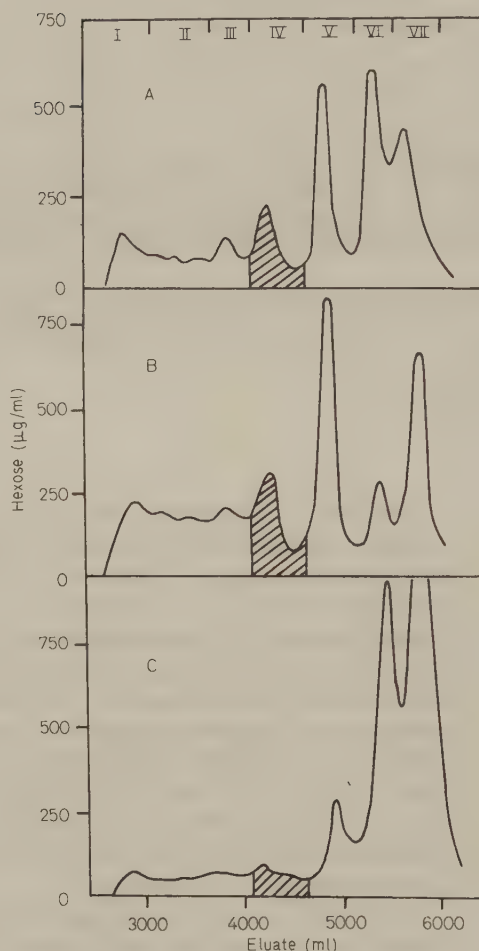


Fig. 1. Gel chromatography on a Sephadex G-25 (fine) column of 24-h urinary ultrafiltrates from a patient with glycogen storage disease type II (A), from a patient with glycogen storage disease type III (B), and from a normal blood group O, secretor (C). Fractions (20 ml) were collected and assayed for total hexose. The material was pooled and concentrated to give fractions I to VII as indicated

alditol acetates; (b) glass capillary column (25 m × 0.25 mm), wall-coated with SE-30 (LKB, Stockholm, Sweden) at a column temperature of 160 °C for partially methylated alditol acetates and at 210–310 °C for permethylated alditol derivatives of di, tri and tetrasaccharides. For gas-liquid chromatography/mass spectrometry a Varian MAT 311 instrument fitted with the same columns as above was used. The mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 1 mA and an ion source temperature of 120 °C. All data were processed by on-line computer system (Spectroscopy 100 Varian MAT). Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. The determinations of muscle glycogen content and the activity of amylo-1,6-glucosidase were performed as described by Öcker-man [13].

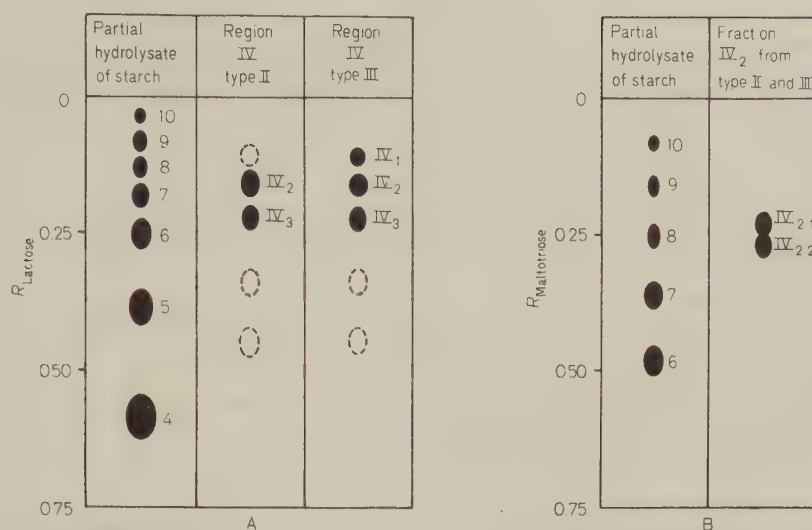


Fig. 2. Fractionation by paper chromatography of (A) gel chromatographic fraction IV and (B) fraction IV₂ from the patients with glycogen storage disease type II and type III. (A) The papers were developed in system a, and stained with a silver dip reagent. The mobilities are expressed as R_{Lactose} values and are compared with those of maltodextrins from a partial hydrolysate of starch with 4 = maltotetraose, 5 = maltopentaose, etc. (B) Fractionation by paper chromatography in solvent b of the compound IV₂ obtained after paper chromatographic fractionation in solvent a. The mobilities are expressed as $R_{\text{Maltotriose}}$ values and are compared with those of maltodextrins (see A)

Table 1. Analytical properties of isolated oligosaccharides

Oligosaccharides	R_{Lactose} values in solvent a	$R_{\text{Maltotriose}}$ values in solvent						Yield	$[\alpha]_D^{20}$
		a	b	c	d	e	f		
								mg/24 h	degrees
Type II: IV _{2.1}	0.16	0.28	0.23	0.16	0.13	0.13	0.17	4.4	+ 169
IV _{2.2}	0.16	0.28	0.27	0.18	0.14	0.13	0.18	6.9	+ 171
IV ₃	0.23	0.39	0.31	0.25	0.22	0.22	0.40	3.5	+ 155
Type III: IV ₁	0.11	0.18	0.17	0.10	0.07	0.07	0.10	11.4	+ 170
IV _{2.1}	0.16	0.28	0.23	0.16	0.13	0.13	0.17	8.2	+ 160
IV _{2.2}	0.16	0.28	0.27	0.18	0.14	0.13	0.18	11.9	+ 158
IV ₃	0.23	0.39	0.31	0.25	0.22	0.22	0.40	9.3	+ 165

Partial Acid Hydrolysis

To about 3 mg of oligosaccharide, dissolved in 3 ml of water, 6 mg of sodium borohydride were added. After at least 2 h, excess borohydride was destroyed by the addition of Dowex 50 (H^+), which was then removed by filtration. Boric acid was removed by evaporation with methanol. The reduced oligosaccharide was then hydrolysed with 0.25 M aqueous sulphuric acid at 100 °C for 1 h, cooled and neutralized with BaCO_3 . The partial hydrolysate was finally reduced with sodium borodeuteride, permethylated [14] and analysed by gas-liquid chromatography/mass spectrometry.

RESULTS

Isolation of Oligosaccharides

Concentrated urinary ultrafiltrates from the two patients were fractionated by gel chromatography on

a Sephadex G-25 column as illustrated in Fig. 1A and B. For comparison, a fractionation of urine from a normal individual is also given (Fig. 1C). The eluted material was pooled as indicated. Region V contains the glucose-containing tetrasaccharide described previously [1,2]. The material in region IV from the two patients was subjected to further purification by zone electrophoresis (pH 1.9). The main carbohydrate-containing material was stationary and was fractionated by preparative paper chromatography (solvent a) (Fig. 2A). Five components were observed in each case of which IV₂ and IV₃ were predominant in the patient with glycogen storage disease type II, components IV₁, IV₂ and IV₃ in the type III patient. Component IV₂ from both patients was further fractionated into IV_{2.1} and IV_{2.2} by repeated preparative paper chromatography (solvent b) (Fig. 2B). Compounds IV_{2.1}, IV_{2.2} and IV₃ from the type II patient and IV₁, IV_{2.1}, IV_{2.2}, and IV₃ from the type III patient were finally passed through a column of Bio

Table 2. Methyl ethers obtained in methylation analysis of reduced oligosaccharides

Retention times t_R of the corresponding alditol acetates are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol

Methylether	t_R values on SE-30	Relative molar proportions						
		type II			type III			
		IV _{2.1}	IV _{2.2}	IV ₃	IV ₁	IV _{2.1}	IV _{2.2}	IV ₃
1,2,3,5,6- <i>O</i> -Me ₅ -Glc-ol	0.62	+	+	+	+	+	+	+
2,3,4,6- <i>O</i> -Me ₄ -Glc	1.00	0.6	2.5	0.6	0.5	0.5	2.2	0.5
2,3,6- <i>O</i> -Me ₃ -Glc	1.50	0.9	2.0	0.9	1.9	1.0	1.9	1.0
2,3,4- <i>O</i> -Me ₃ -Glc	1.62	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2,3- <i>O</i> -Me ₂ -Glc	2.31	—	0.8	—	—	—	0.6	—

^a The yield of this component was low due to its high volatility.

Gel P-2. Hexose assay of eluted fractions revealed symmetrical peaks. The carbohydrate-containing fractions were pooled and the material lyophilized. Yields are given in Table 1.

Characterization of the Oligosaccharides

The oligosaccharides IV₁ (from the type III patient) and IV_{2.1}, IV_{2.2}, and IV₃ from both patients were homogeneous as judged by paper chromatography in six solvent systems. Chromatographic mobilities, yields, and optical rotations are given in Table 1. Sugar analyses showed that D-glucose constituted between 95 and 100% of the dry weight of all the compounds above.

Methylation analysis of the reduced (NaB²H₄) oligosaccharides gave the results shown in Table 2.

In order to obtain information about the sequential order of the (1→4) and (1→6)-linked D-glucose residues, the reduced (NaBH₄) oligosaccharides were partially hydrolysed, reduced (NaB²H₄) and methylated as described in Experimental Procedure. The resulting permethylated oligosaccharide alditols were then analysed by gas-liquid chromatography/mass spectrometry (Fig. 3).

Identification of Permethylated Di, Tri and Tetrasaccharides after Partial Hydrolysis

The oligosaccharides studied are composed of α(1→4) or α(1→6)-linked D-glucose residues. In the partial acid hydrolysis of the reduced (NaBH₄) oligosaccharides a mixture of smaller oligosaccharides was obtained. This mixture was analysed, after reduction (NaB²H₄) and permethylation, by gas-liquid chromatography/mass spectrometry. The characterization of the permethylated oligosaccharide alditols was based upon their mass spectra and, when authentic samples were available, also by comparison of gas-liquid chromatography retention times.

Permethylated Disaccharide Alditols. Glc(1→4)Glc*, Glc(1→6)Glc* and their analogues with a deuterium label at C-1 of the alditol part can be formed. Their retention times on gas-liquid chromatography are given in Table 3. The linkage type was determined by inspection of the fragments formed by α-cleavage of the alditol chain (Fig. 4). A deuterium label at C-1 of the alditol unit was determined by the same fragmentation or by the fragments representing the alditol unit (Fig. 4).

Permethylated Trisaccharide Alditols. The four permethylated trisaccharide alditols Glc(1→4)Glc(1→4)Glc*, Glc(1→6)Glc(1→4)Glc*, Glc(1→4)Glc(1→6)Glc* or Glc(1→6)Glc(1→6)Glc* and their analogues with a deuterium label at C-1 of the alditol part can be formed. The gas-liquid chromatography retention times (Table 3) were determined, using authentic samples, for all compounds except Glc(1→4)Glc(1→6)Glc*. The linkage to the alditol part and deuterium label at C-1 of the alditol were determined as for permethylated disaccharide alditols. The linkage type to the middle sugar unit was determined by inspection of the alditol fragments b since the intensity ratios were different depending on the linkage type. This difference must be due to different rates of elimination of methanol and/or stability of resulting fragments (Fig. 5).

Permethylated Tetrasaccharide Alditols. The tetrasaccharides were characterized by the same method as for the trisaccharides, i.e. the linkage type to both the internal sugar residues was determined by inspection of the alditol fragments c and bc. The intensity ratios between the alditol fragments c, (c-32), (c-2×32) and between bc, (bc-32), (bc-2×32) were similar, about 16:4:1 for a 4-linked sugar residue and about 1:5:2 for a 6-linked sugar residue. These differences were found for permethylated alditols of Glc(1→4)Glc(1→4)Glc(1→4)Glc*, Glc(1→6)Glc(1→6)Glc(1→6)Glc* and Glc(1→6)Glc(1→4)Glc(1→4)Glc* which were available as authentic samples. One example is shown in Fig. 6. Gas-liquid chromatog-

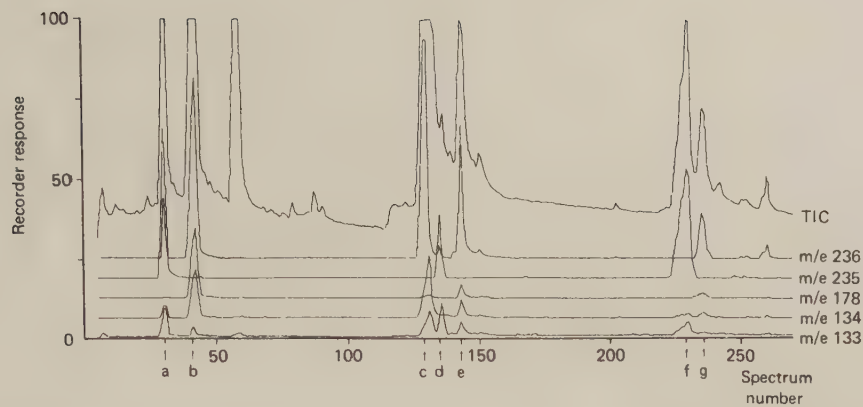


Fig. 3. Multiple ion tracing and mass spectra of permethylated oligosaccharide alditols obtained from a partial hydrolysate of component IV₃. (TIC = total ion current)

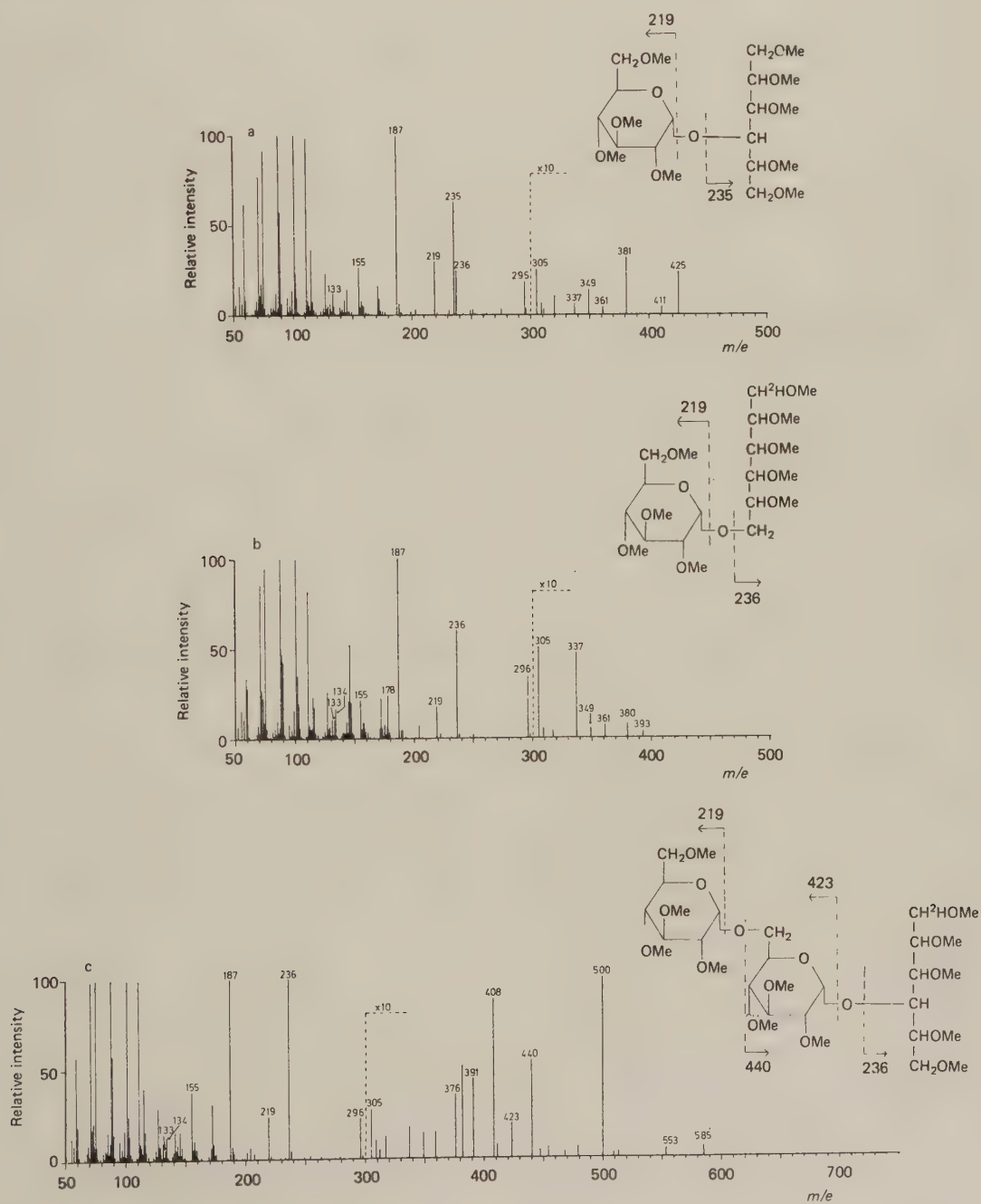


Fig. 3a-c

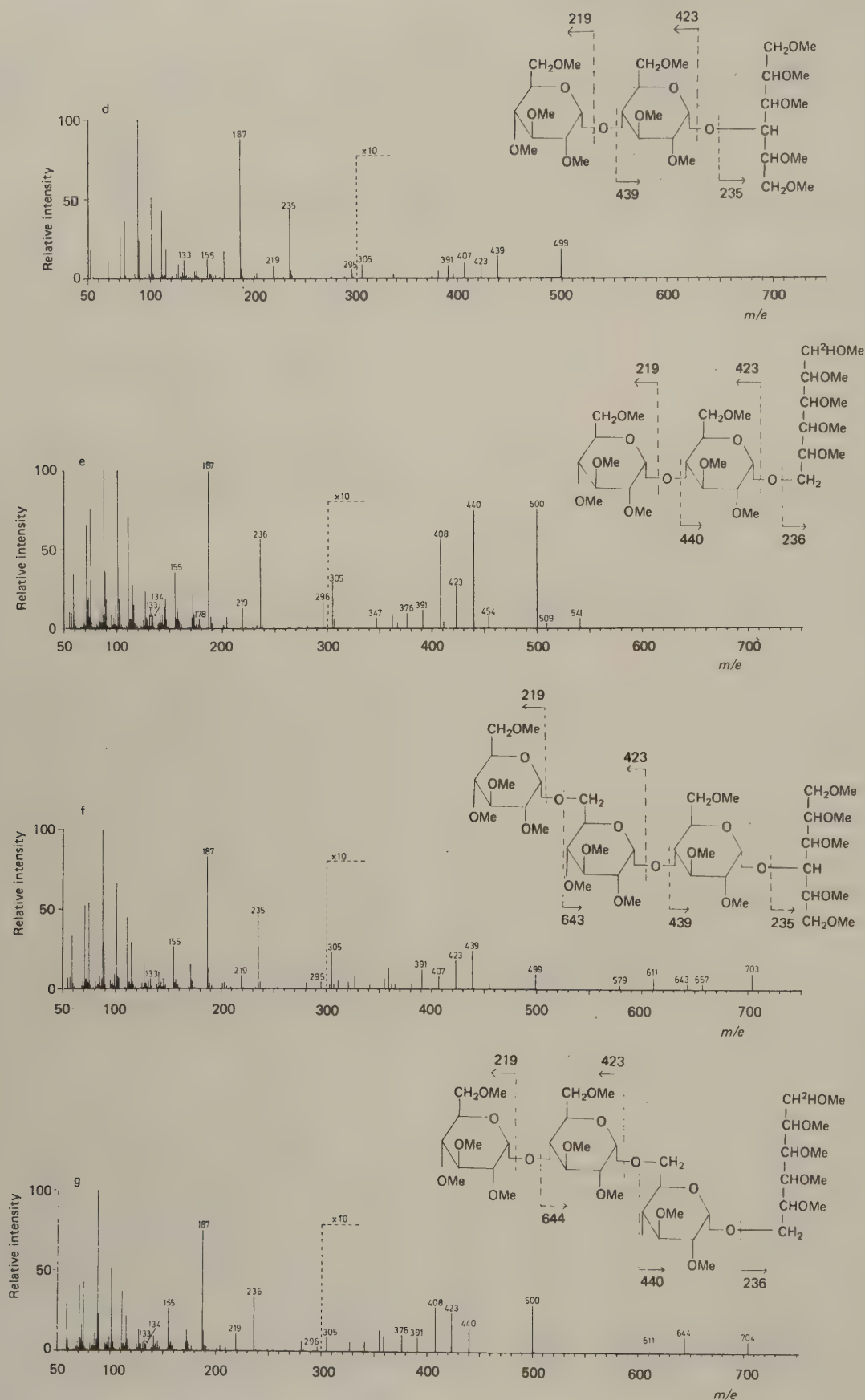


Fig. 3d—g

Table 3. *Oligosaccharides obtained in the partial hydrolysis studies*

Retention times for the corresponding permethylated alditol derivatives are given. They were obtained with temperature programme 210–330° C (8° C/min). n.d. = not determined

Oligosaccharides	Notation in Fig. 3	IV ₁	IV _{2,1}	IV _{2,2}	IV ₃	Retention time
						min
Glc(1–4)Glc	a	+	+	+	+	6.3
Glc(1–6)Glc	b	+	+	+	+	7.2
Glc(1–6)Glc(1–4)Glc	c	+	+	+	+	13.4
Glc(1–4)Glc(1–4)Glc	d	+	+	+	+	13.5
Glc(1–4)Glc(1–6)Glc	e	+	–	+	+	14.4
Glc(1–6)Glc(1–6)Glc	–	–	+	–	–	14.6
Glc(1–6)Glc(1–4)Glc(1–4)Glc	f	+	–	n.d.	+	23.3
Glc(1–6)Glc(1–6)Glc(1–4)Glc	–	–	+	n.d.	–	23.5
Glc(1–4)Glc(1–6)Glc(1–6)Glc	g	–	–	n.d.	+	24.2
Glc(1–4)Glc(1–4)Glc(1–4)Glc	–	–	+	n.d.	–	24.5

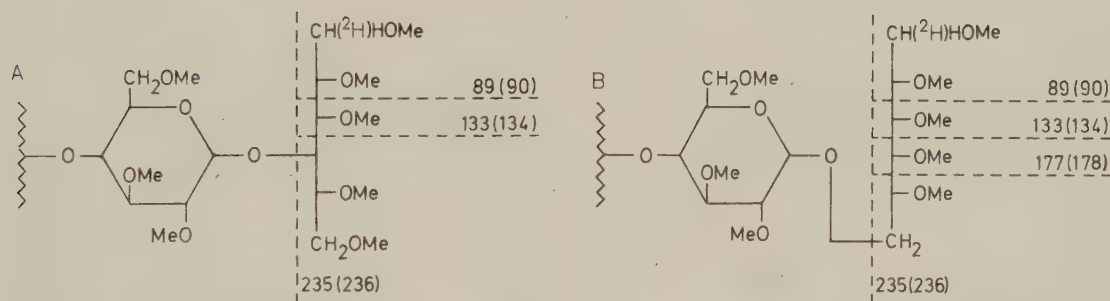


Fig. 4. Some fragments of the permethylated oligosaccharide alditols. (A) (1→4)-linked reducing end. (B) (1→6)-linked reducing end

raphy retention times (Table 3) were determined by authentic samples only for Glc(1–4)Glc(1–4)Glc(1–4)Glc*, Glc(1–4)Glc(1–4)Glc* and Glc(1–6)Glc(1–6)Glc(1–6)Glc* due to lack of standards.

Structures of Isolated Oligosaccharides

Component IV₁. Sugar analysis, optical rotation and methylation analysis of component IV₁ demonstrate that it is a linear octasaccharide composed of a non-reducing α -D-glucose residue, four α (1→4)-substituted D-glucose residues, two α (1→6)-substituted D-glucose residues, and one 4-substituted D-glucose residue as reducing terminal. Partial hydrolysis and reduction of reduced (NaBH₄) component IV₁ yielded the di, tri, and tetrasaccharides Glc(1–4)Glc*, Glc-

(1–4)Glc, Glc(1–6)Glc, Glc(1–4)Glc(1–4)Glc*, Glc(1–6)Glc(1–4)Glc, Glc(1–4)Glc(1–6)Glc, Glc(1–6)Glc(1–4)Glc(1–4)Glc* and Glc(1–6)Glc(1–4)Glc(1–4)Glc. The two tetrasaccharides were formed in equal amounts and if it is assumed that the tetrasaccharides are formed by cleavage of only one bond in IV₁, only one possible structure exists for IV₁: Glc(1–6)Glc(1–4)Glc(1–4)Glc(1–4)Glc(1–6)Glc(1–4)Glc(1–4)Glc*.

Component IV_{2,1}. Sugar analysis, optical rotation, and methylation analysis of component IV_{2,1} from both patients show that it is a hexasaccharide consisting of a non-reducing terminal α -D-glucose residue, two α (1→4)-substituted D-glucose residues, two α (1→6)-substituted D-glucose residues and a 4-substituted D-glucose residue as reducing terminal. Partial hydrolysis and reduction of reduced (NaBH₄) com-

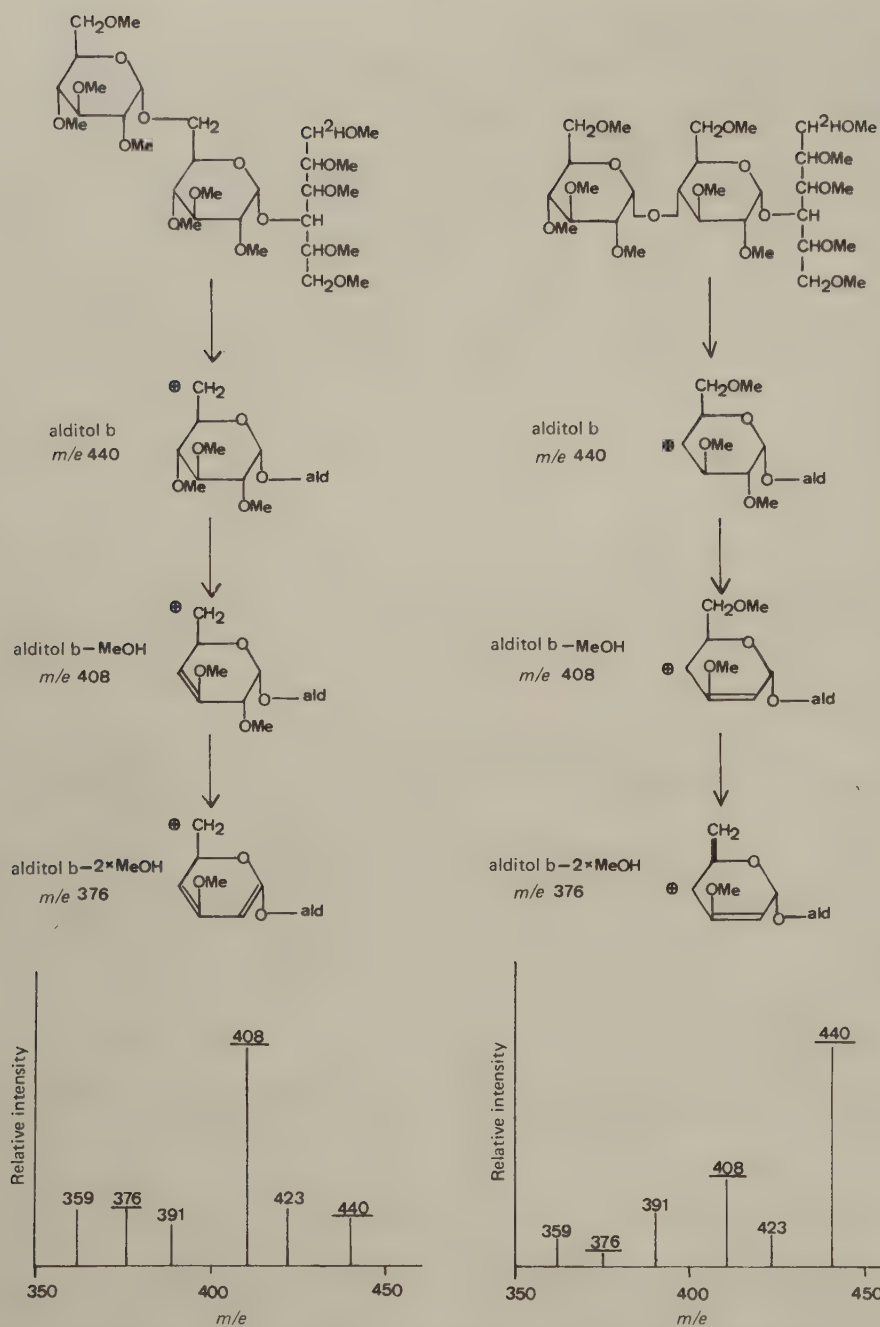


Fig. 5. Determination of one intrachain linkage by comparison of elimination of methanol from the alditol fragment *b*

pound IV_{2.1} yielded the following di, tri, and tetrasaccharides: Glc(1→4)Glc*, Glc(1→4)Glc(1→4)Glc*, Glc(1→4)Glc(1→4)Glc(1→4)Glc*, Glc(1→4)Glc, Glc(1→6)Glc(1→4)Glc, Glc(1→6)Glc(1→6)Glc(1→4)Glc, Glc(1→6)Glc, and Glc(1→6)Glc(1→6)Glc. Only the structure Glc(1→6)Glc(1→6)Glc(1→4)Glc(1→4)Glc(1→4)Glc* is compatible with the oligosaccharide pattern obtained in the partial hydrolysate.

Component IV_{2.2}. Sugar analysis, optical rotation and methylation analysis of IV_{2.2} show that it is a heptasaccharide with two non-reducing terminal α-

D-glucose residues, two α(1→4)-substituted D-glucose units, one α(1→6)-substituted D-glucose unit, one branched D-glucose unit substituted in the 1, 4 and 6 positions and finally a reducing 4-substituted D-glucose unit. From these results a large number of structures can be constructed.

Component IV₃. Sugar analysis, optical rotation, and methylation analysis showed that IV₃ is a hexasaccharide composed of a non-reducing terminal α-D-glucopyranose residue, two α(1→4)-substituted D-glucose residues, two α(1→6)-substituted D-glucose residues

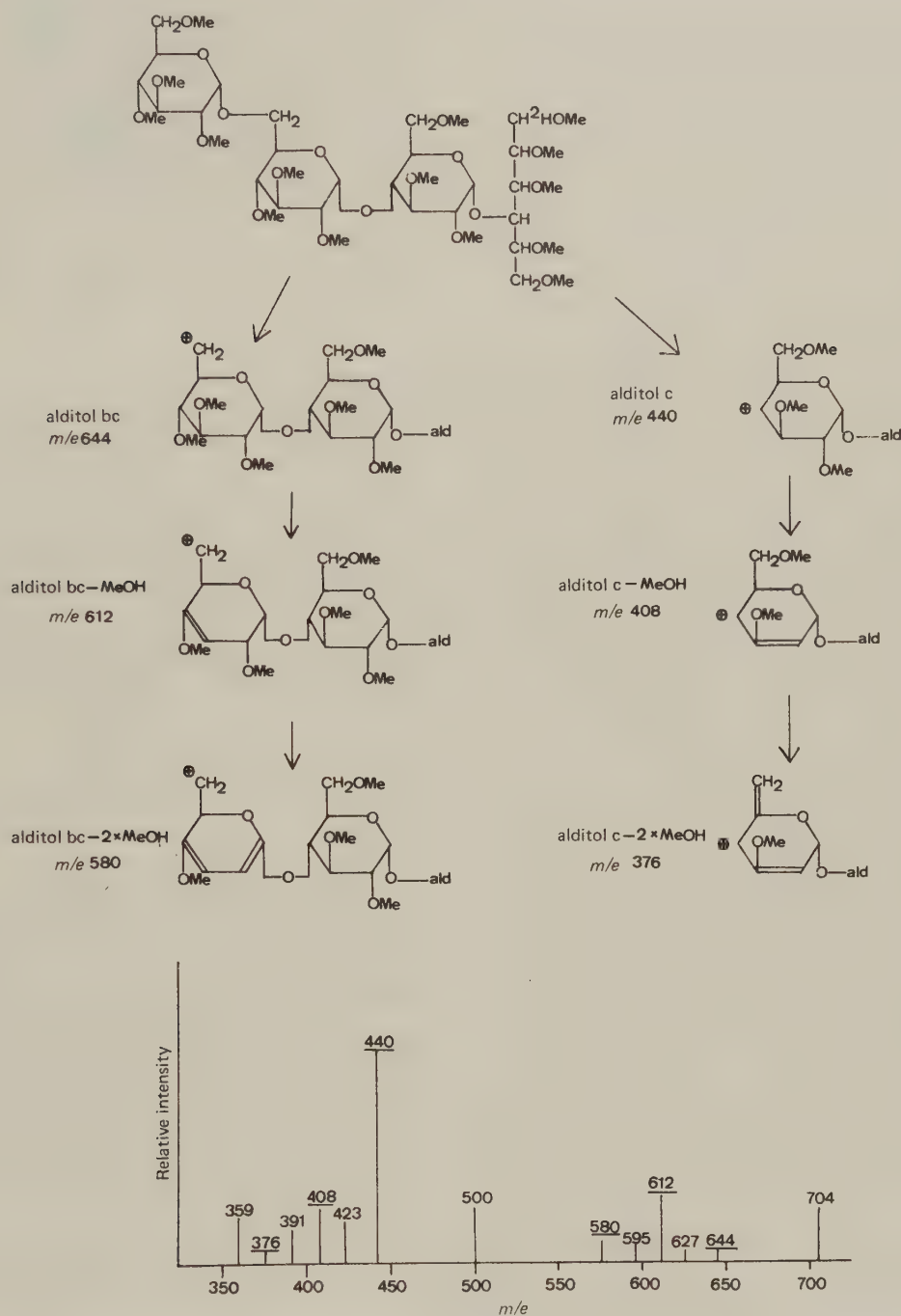


Fig. 6. Determination of two intrachain linkages by comparison of elimination of methanol from the alditol fragments *c* and *b*

and a 4-substituted D-glucose residue as reducing terminal. Partial hydrolysis and reduction of reduced IV₃ yielded the following di, tri, and tetrasaccharides: Glc(1-4)Glc*, Glc(1-4)Glc(1-4)Glc*, Glc(1-6)Glc(1-4)Glc(1-4)Glc*, Glc(1-4)Glc, Glc(1-6)Glc(1-4)Glc, Glc(1-4)Glc(1-6)Glc(1-6)Glc, Glc(1-6)Glc and Glc(1-4)Glc(1-6)Glc. Only the structure Glc(1-4)Glc(1-6)Glc(1-6)Glc(1-4)Glc(1-4)Glc* can accommodate the above data.

DISCUSSION

The origin of the isolated oligosaccharides is not known. Their structures, however, strongly indicate that glycogen is the main source. This is also supported by the fact that glycogen is the only substance known to accumulate in both patients studied. The excretion rate of these new larger oligosaccharides is considerably less than that of the main metabolite (Glc)₄

[2] excreted in urine by these patients. In contrast to the excretion of (Glc)₄, the total yield of larger oligosaccharides is higher in the type III patient and in addition one oligosaccharide (an octasaccharide) is exclusive for this patient. No conclusive differences in structures were observed in those compounds that appeared common to both patients.

Patients with glycogen storage disease type II have been shown to accumulate glycogen with apparently normal structure in their lysosomes [3,15], the average length of the carbohydrate chains being in the range of 10–14 glucose residues. It has also been suggested that the internal part of the molecule is more branched than the outer part [16,17]. Patients with glycogen storage disease type III on the other hand accumulate their glycogen in the cytoplasm and this glycogen has been shown to be of 'limit dextrin' type with shorter outer chains [4]. Detailed information about the internal structure in these two types of glycogen is lacking. The structures of the isolated oligosaccharides of six, seven and eight units indicate that they are derived from the more branched internal parts of glycogen, since all of them contain two (1→6) linkages, and in two of the oligosaccharides (1→6) linkages are adjacent. It cannot be ascertained, however, if all these (1→6) linkages derive from branch points in the original glycogen molecule, since it is not known if non-branching α(1→6) linkages occur. A more detailed investigation of the internal structure of amylopectin has been performed [18] and the suggested structure of this molecule contains all the elements represented by these urinary oligosaccharides. If glycogen is the source of all these urinary compounds it must be assumed that they represent 'limit oligosaccharides' obtained after digestion of glycogen mainly by serum and urine amylase.

Finally it cannot be ruled out that some of these compounds are biosynthetic rather than degradation products. (Glc)₄ for instance, which is the most abundant metabolite, is an integral part of all the

larger oligosaccharides, might serve as substrate for different serum glycosyltransferases.

The oligosaccharide patterns obtained from the two patients with glycogen storage disease type II and type III respectively showed only minor differences and can not therefore serve as a diagnostic tool for differentiation between these two diseases.

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G. Lennartson, A. Lundblad, J. Lundsten, and S. Svensson, Institutionen för Klinisk Kemi, Lunds Universitet, Lasarettet, S-221 85 Lund, Sweden

A. Häger, Barnkliniken, Regionssjukhuset, S-581 85 Linköping, Sweden

AN α -L-FUCOPYRANOSYL-MYO-INOSITOL IN NORMAL HUMAN URINE

Gudrun Lennartson and Arne Lundblad

Department of Clinical Chemistry, University Hospital,
S-221 85 Lund, Sweden

Bengt Lindberg and Jörgen Lönnngren

Department of Organic Chemistry, Arrhenius Laboratory,
University of Stockholm, S-104 05 Stockholm, Sweden

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Summary: An α -L-fucopyranosyl-myo-inositol has been isolated from normal urine of ABH-secreters. The compound was also present in small amounts in the urine of ABH-non-secreters. After ingestion of 20 g of myo-inositol, two secretors increased their excretion of α -L-fucopyranosyl-myo-inositol three and thirteen times respectively. The same myo-inositol diet did not give rise to any increased excretion of α -L-fucopyranosyl-myo-inositol in the urine of two non-secreters.

INTRODUCTION

Galactinol (1-L-1-O- α -D-galactopyranosyl-myo-inositol) was the first glycoside of myo-inositol to be isolated (1). It was found to occur in the juice of the sugar beet. The structure was elucidated by Kabat et al. (2). From rape-seed meal a trisaccharide identified as α -D-Galp-(1 $\rightarrow$ 6)- α -D-Galp-(1 $\rightarrow$ 1)-myo-inositol was isolated (3). Two other glycosides, β -D-Gal-(1 $\rightarrow$ 6)-myo-inositol from rat tissues (4, 5, 6) and β -D-Man-(1 $\rightarrow$ 6)-myo-inositol from *Saccharomyces cerevisiae* (7), have also been described. The first glycoside of myo-inositol in human material was found in 1970 when one of us reported on the presence of a fucosyl-myo-inositol in the urine of ABH-secreters (8). We now present further structural studies of this substance. A gas chromatographic-mass spectrometric method (9, 10) has been used to determine the urinary excretion rate of α -L-fucopyranosyl-myo-inositol in two secretors and two non-secreters on myo-inositol diet.

EXPERIMENTAL PROCEDURE

Materials. Pooled urine (5 liters) was collected from ten healthy ABH-secretors without any dietary restrictions. Urine samples produced during six hours following 12 hours of starvation and ingestion of 20 g of myo-inositol was collected from four individuals, two secretors (blood groups A and O) and two non-secretors (blood groups A and B). Control samples were collected the same way, but omitting the myo-inositol. 30 ml of saturated phenyl mercuric nitrate solution was added per liter of urine to prevent bacterial growth. Galactinol was a gift from Dr. Clinton Ballou, University of California, Calif., U.S.A.

Isolation Procedure. The pooled urine samples were purified by ultra-filtration, concentration and gel chromatography (Sephadex G-25) as previously described (11). The disaccharide region (VI) was pooled, deionized by passage through Dowex 50 (H^+) and Bio-Gel AG3-X4A (OH^-), and fractionated by preparative descending paper chromatography on Whatman No. 1 paper (butan-1-ol - pyridine - water, 3:2:1.5, v/v) (double development) (Fig. 1). Fraction VI b, known to contain the fucosyl-myo-inositol (8), was further purified by paper chromatography using first ethyl acetate - acetic acid - water (3:1:1, v/v) (48 hours) and then propan-1-ol - ethyl acetate - water (6:1:3, v/v) (24 hours). The fucosyl-myo-inositol obtained was finally purified by passage through a Bio-Gel P-2 column. Approximately 2 mg of purified fucosyl-myo-inositol per liter of urine was isolated.

Analytical Methods. Sugar analysis was performed by gas liquid chromatography (12) and mass spectrometry (13). Optical rotations were determined using a Perkin-Elmer 2411 polarimeter. Methylation analysis was performed as previously described (14). Analysis of permethylated disaccharide was done using a glass capillary column (25 m x 0.25 mm) wall-coated with SE-30 (LKB, Stockholm, Sweden). The separations were performed at 200°C. For gas liquid chromatography-mass spectrometry, the same column as above was

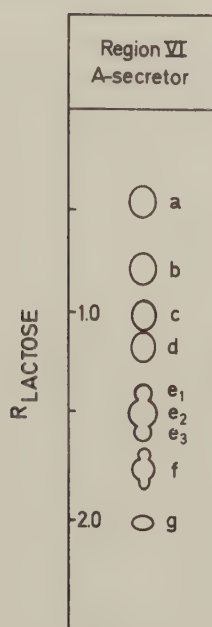


Fig. 1. Paper chromatography of gel chromatographic fraction VI (disaccharide region) from urine of a starved A-secretor. The paper was developed in butan-1-ol - pyridine - water (3:2:1.5, v/v).

introduced directly into the ion source of a Varian MAT 311 A combined gas liquid chromatography-mass spectrometry instrument. The mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 3 mA and an ion source temperature of 120°C. The data were processed by on-line computer system (Spectrosystem 100 MS, Varian MAT). The procedure for quantitation of fucosyl-myo-inositol was essentially the same as previously described for other urinary oligosaccharides (9, 10, 15). Cellobiose was used as an internal standard and the retention time of its reduced and permethylated derivative was set to 1 (T=1). A linear response ($k = 1.6$) was obtained within the range 25-150 μ g. The identification of the fucosyl-myo-inositol in the different urine samples was based on identical retention time and mass spectrum as compared to those of an authentic sample.

RESULTS AND DISCUSSION

Characterization. Purified fraction VI b, $[\alpha]_D^{20} -70^\circ$ (c. 1.35, water), on acid hydrolysis yielded equimolar amounts of fucose and myo-inositol. The products in the neutralized hydrolysate were treated with sodium borohydride and acetylated and the components identified as fucitol pentaacetate and myo-inositol hexaacetate by gas liquid chromatography (co-chromatography with authentic samples) and mass spectrometry. Isomeric 6-deoxyhexitol pentaacetates and inositol hexaacetates are well separated from those obtained.

In a hydrolysed sample, the optical rotation should be due only to the fucose, as myo-inositol is optically inactive. A hydrolysate of 0.64 mg glycoside in 1 ml 0.125 M sulfuric acid showed $[\alpha]_D^{20} -0.027^\circ$ from which the value $[\alpha]_D^{20} -84^\circ$ for the fucose could be calculated. This is in reasonably good agreement with the value for L-fucose, $[\alpha]_D^{20} -76^\circ$, and demonstrates that the fucose moiety in the glycoside has the L-configuration.

The fully methylated glycoside gave a single peak on gas liquid chromatography ($T_{\text{cellobiitol}} = 1.14$) and its mass spectrum is given in Fig. 2. Strong fragments of m/e 233 and 201 derive from the inositol moiety as indicated in Fig. 3a. A strong J_1 fragment (Fig. 3b), m/e 293, was also obtained. Only weak fragments in the A series, e.g. A_1 (m/e 189), were observed, and the reason for this is not understood. Mass spectrometry of fully methylated galactinol was, however, perfectly analogous. Hydrolysis of the methylated glycoside yielded 2,3,4-tri-O-methyl-L-fucose and a penta-O-methyl-myo-inositol, as evident by gas liquid chromatography-mass spectrometry of the product obtained after borohydride reduction and acetylation. The results above consequently demonstrate that the L-fucosyl group in the glycoside is pyranosidic and, from the negative value of the optical rotation, α -linked.

The O-acetyl-penta-O-methyl-myo-inositol obtained from the glycoside was chromatographically distinguishable from the corresponding derivative obtained on methylation analysis of galactinol. The α -L-fucopyranosyl group is therefore not linked to O-1 (O-3) of myo-inositol. The nature of this linkage is subject to further studies.

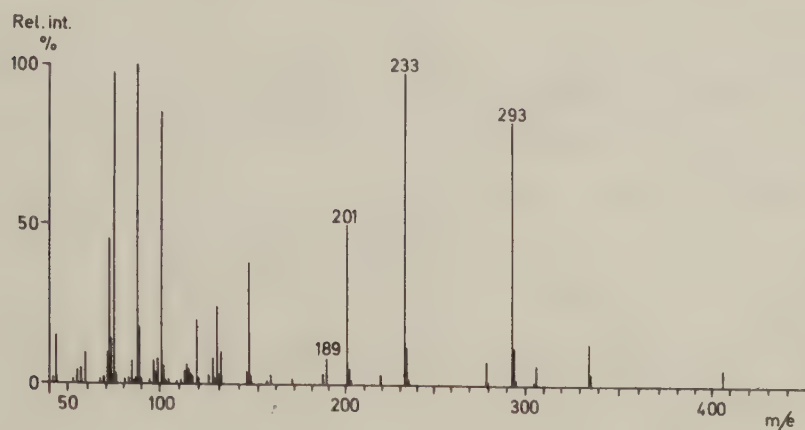


Fig. 2. Mass spectrum of α -L-fucopyranosyl-myo-inositol as its permethylated derivative.

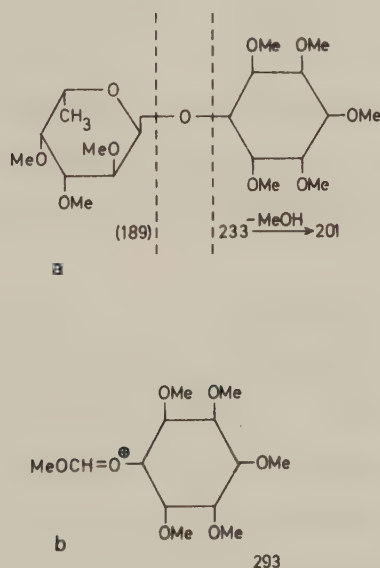


Fig. 3. a) Primary fragmentation of α -L-fucopyranosyl-myo-inositol as its permethylated derivative.

b) J_1 fragment m/e 293.

Quantitation. The content of fucosyl-myo-inositol in the disaccharide region of individual secretor and non-secretor urine samples was determined by gas liquid chromatography-mass spectrometry (Table I). Approximately

Table I

Quantitative determinations of α -L-fucopyranosyl-myo-inositol
in different urine samples

	n	α - <u>L</u> -Fucopyranosyl- <u>myo</u> -inositol mg/24 h	
		Starved	Oral <u>myo</u> -inositol
A-secretor	1	1.2	16.0
O-secretor	1	3.2	10.4
Non-secretor	2	0.4	0.4

2-3 mg per 24 h was excreted by the starved secretors. A three and thirteen fold increased excretion respectively was observed after ingestion of myo-inositol. A small amount (approximately 0.4 mg/24 h) was excreted by two non-secretors.

Ingestion of 20 g of myo-inositol did not affect the excretion rate of fucosyl-myo-inositol in the two non-secretors. The origin and physiological role of this new component is not known. It has not as yet been possible to demonstrate the addition of fucose to either inositol or phosphatidyl inositol using α -L-fucosyltransferases from a variety of human sources (16), and therefore it is difficult to explain why the excretion rate of α -L-fucopyranosyl-myo-inositol is secretor dependent.

ACKNOWLEDGEMENTS

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ON THE ORIGIN OF URINARY MYOINOSITOL AND SOME OLIGOSACCHARIDES
DURING PREGNANCY AND LACTATION

Gudrun Lennartson, Bo S. Lindberg, Arne Lundblad, and
Tord Löfstrand

Department of Clinical Chemistry, University Hospital,
S-221 85 Lund, Department of Obstetrics and Gynaecology,
Central Hospital, S-721 89 Västerås, and Department of
Gynaecology, Central Hospital, S-541 85 Skövde, Sweden

Summary

The urinary excretion of free myoinositol during pregnancy and lactation has been determined and related to the excretion of two pregnancy dependent glycosides of myoinositol. Free myoinositol was also determined in amniotic fluid at different times during pregnancy. The amount of free myoinositol and other, glucose-containing oligosaccharides was estimated in an ultrafiltered water extract of placenta. No myoinositol glycosides were detected despite high levels of myoinositol.

Free myoinositol appears to be a requirement for the formation of the glycosides of myoinositol, but where this biosynthesis takes place is unclear. The increased excretion of glyco-gen degradation products during pregnancy, however, can be explained by the high content of these compounds in placenta.

Introduction

The excretion pattern of different oligosaccharides in urine of pregnant and lactating women has been studied (1). Additional information was obtained by studying a larger number of individuals and by using a radioimmunoassay method for the determination of two oligosaccharides, lacto-N-difucohexaose I (2) and a glucose-containing tetrasaccharide (3). It was found that during pregnancy all oligosaccharides reach a maximum level during the last trimester. After delivery some oligosaccharides decrease or disappear and are thus pregnancy dependent. Others, namely those with a lactose, lacto-N-tetraose or lacto-N-neotetraose backbone are also excreted in high amounts during lactation and are probably derived from the mammary gland.

There are at least two groups of pregnancy-dependent oligosaccharides; some glycosides of myoinositol and some fragments of glycogen. It is not known if the excretion of these oligosaccharides reflects the function of the placenta or the fetus, or both.

We have therefore tried to find out more about the origin of the glycosides of myoinositol and the glucose-containing oligosaccharides and have compared their excretion with that of free myoinositol. We have also determined the amount of free myoinositol and different oligosaccharides in both amniotic fluid and the placenta.

Materials and Methods

Urine. One or more urine samples were collected from 13 non-pregnant and from 134 pregnant or lactating women. 24 h urine samples were also collected from 4 women (I.W., E.H., C.L., and G.F.) at approximately fortnightly intervals during their pregnancy and subsequent lactation. All urine samples were stored at -20°C .

Amniotic fluid. About 5 ml of amniotic fluid was taken through transabdominal amniocentesis from 59 different women at various times during the 2nd and 3rd trimester. The reason for amniocentesis in the 2nd trimester was prenatal diagnosis of chromosomal aberrations. All 59 women included in this material had normal pregnancies and gave later birth to normal children.

Placentas. Three macroscopically normal placentas were collected immediately after delivery. They were each thoroughly perfused with 2 litres of ice-cold saline and stored at -20°C . A 20% (w/v) homogenate was made in re-distilled water using a Dounce tissue homogenizer. The sample was centrifuged at $32,000 \times g$ for 20 min and the resulting supernatant was ultrafiltered using an Amicon ultrafiltering device (Filter PM 30). The ultrafiltrate was concentrated by rotatory evaporation.

Gel chromatography. A Sephadex G-15 column (5 x 130 cm; void volume 900 ml) eluted with distilled water at a flow rate of 70 ml/h was used.

Paper chromatography. Whatman No. 3 papers were used in the following systems: (a) ethyl acetate:acetic acid:water (3:1:1, v:v:v), (b) ethyl acetate:pyridine:acetic acid:water (5:5:1:3, v:v:v:v), and (c) ethyl acetate:pyridine:water (2:1:2,

v:v:v, upper phase). After development the papers were stained with a silver dip reagent (4).

Analytical methods. Total hexose in eluates from the gel chromatographic column was assayed by the anthrone reaction (5). Creatinine was determined by the method of Løken (6). Myoinositol and other monosaccharides were determined in desalted urine or amniotic fluid as their peracetylated derivatives by gas-liquid chromatography (7) using α -phenyl-D-glucopyranoside as internal standard. All samples were analyzed twice and the precision of the method was high (coefficient of variation <5%). Quantitation of different oligosaccharides was performed by combined gas-liquid chromatography-mass spectrometry of reduced and permethylated derivatives as described previously (8,9). Sugar analyses were done after hydrolysis (90% aqueous formic acid for 5 h at 100°C followed by 0.25 M aqueous sulphuric acid for 18 h at 100°C) by gas-liquid chromatography-mass spectrometry (7,10).

Results and Discussion

Determination of free myoinositol in urine

The mean value for non-pregnant women was 1.9 mg myoinositol/100 mg creatinine (Table I), which is in reasonably good agreement with Pfaffenberger *et al.* (11), who found a mean value of 3.3 mg myoinositol/100 mg creatinine as compared to 220 mg/100 mg creatinine in newborn infants. During pregnancy there is an increased excretion of myoinositol, with the highest values usually being obtained in the third trimester. There is,

TABLE I

Excretion rate of myoinositol expressed as mg myoinositol/100 mg creatinine in urine of non-pregnant, pregnant, and lactating women

	Number of women	Mean value	Range
Non-pregnant	13	1.9	0.6-4.3
1st trimester	31	3.7	1.5-8.9
2nd trimester	36	4.7	2.0-16.3
3rd trimester	58	7.0	2.0-31.2
Post partum	11	1.3	0.9-2.7

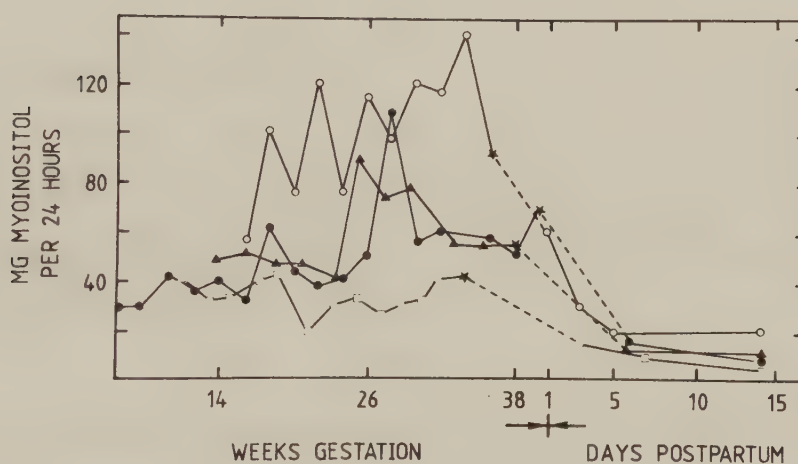


Fig. 1. Urinary excretion of myoinositol (mg/24 h) in four women followed approximately biweekly throughout their pregnancies and subsequent lactation.

○—○ O Le^{a-b+}; ●—● A Le^{a-b+}; □—□ A Le^{a-b-};
 ▲—▲ A Le^{a-b+}; ★ partus.

however, a large individual variation which is illustrated by the large ranges for each trimester. Immediately after partus there is a sudden decrease in excretion rate, which is illustrated in Fig. 1. Within the first 2-5 days post partum, the excretion reached non-pregnancy levels. Fig. 1 also illustrates that the excretion rate values obtained during pregnancy for

TABLE II
Structure of oligosaccharides¹⁾

Trivial name (abbreviation)	Structure
Fucosylgalactosylinositol (FGIno)	Gal β 1- <u>myo</u> -inositol 2 Fucal
Difucogalactosylinositol (DFGIno)	Gal β 1- <u>myo</u> -inositol 2 O Fucal Fucal
Glucose tetrasaccharide (Glc) ₄	Glc α 1-6Glc α 1-4Glc α 1-4Glc
Lacto-N-difucohexaose I (LNDI)	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc 2 4 Fucal Fucal

¹⁾ Fucose has the L-configuration, all other monosaccharides shown have the D-configuration.

all four women were always more than three times higher than values obtained during lactation. With one of the women the highest value during pregnancy was 11 times higher than during lactation. No correlation could be found between myoinositol level during pregnancy and ABO- or Lewis-blood group of the women. Also the birthweight of the infant was independent of myoinositol excretion rate of the mother.

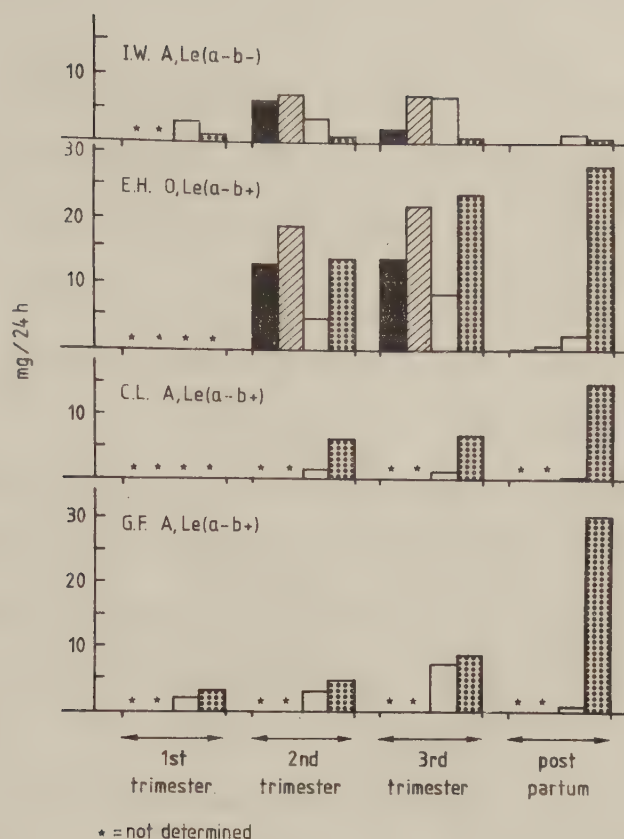


Fig. 2. Excretion rates of FGIno (■), DFGIno (▨), (Glc)₄ (□), and LNDI (▤) in urines of the four women I.W. (A Le^{a-b-}), E.H. (O Le^{a-b+}), C.L. (A Le^{a-b+}), and G.F. (A Le^{a-b+}) during pregnancy and subsequent lactation. The bars represent mean values for at least five different samples in each trimester and post partum.

Excretion rates of four different oligosaccharides in urines from the four women studied above (Fig. 1)

The excretion rates of two glycosides of myoinositol (FGIno and DFGIno), the glucose tetrasaccharide (Glc)₄ and lacto-N-difucohexaose I (LNDI) (Table II), in urines from the four women I.W., E.H., C.L., and G.F. have been compiled in Fig. 2. Each bar represents a mean value for at least five different samples in each trimester and post partum. Values for (Glc)₄ and LNDI are taken from previous studies on the same urine samples from the same women (2,3).

(Glc)₄ and the two glycosides of myoinositol are pregnancy specific and decrease or disappear in urine collected post partum.

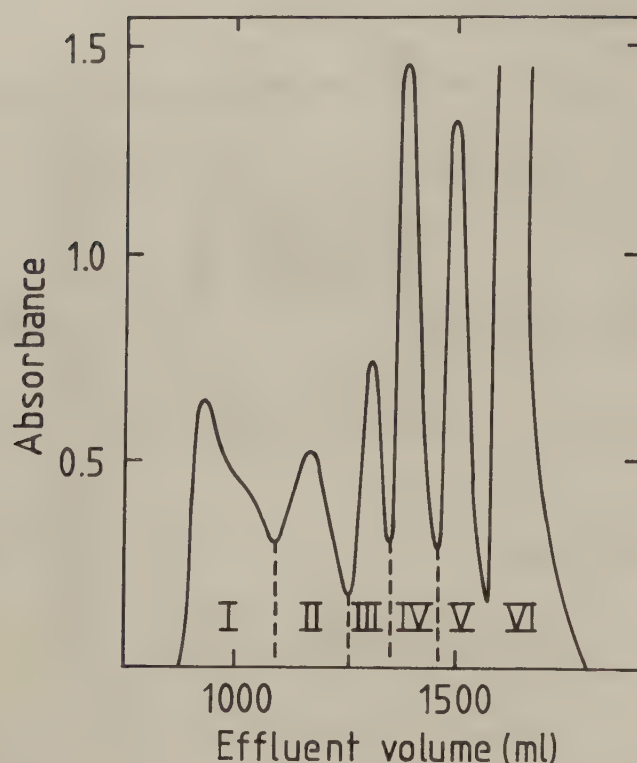


Fig. 3. Gel chromatogram (Sephadex G-15) of the low molecular weight carbohydrate-containing material in a water extract of human placenta. 10 ml of concentrated extract was applied to the column (5 x 130 cm; void volume = 900 ml) and eluted with distilled water.

The LNDI on the other hand which is only present in urine of the three women with blood type Le^{a-b+} is excreted in highest amounts post partum.

Myoinositol and oligosaccharide content in placenta

The concentrated, ultrafiltered water extract of a whole placenta was examined by gel chromatography (Sephadex G-15) and eluted fractions were analyzed for total hexose (Fig. 3). Six carbohydrate fractions (I-VI) were obtained as indicated. Sugar analyses revealed glucose as the main constituent in all fractions, except fraction V in which myoinositol was the dominating component.

Gas-liquid chromatography of reduced and peracetylated fractions V and VI showed that all the myoinositol in placenta was present in the free form. Reduced and permethylated fractions III, IV, and V were analyzed by combined gas-liquid chromatography-mass spectrometry and fractions I to VI by paper chromatography using three different systems (a, b, and c). The results of these studies are summarized in Table III. The qualitative analyses were repeated with two additional placentas and similar results were obtained.

TABLE III

Main compounds identified in water extract of human placenta

Gel chromatographic fraction	Compound	Approximate amount (mg) in whole placenta
I	Maltodextrins	n.d.
II	Maltodextrins (M_5 - M_9)	n.d.
III	Maltotetraose	9
	Glucose tetrasaccharide	1
IV	Maltotriose	26
V	Maltose	5
	<u>Myoinositol</u>	24
VI	Glucose	3

TABLE IV

Myoinositol in amniotic fluid ($\mu\text{g/ml}$)

	n	mean	range
Second trimester	29	22.6	4.4-38.7
Third trimester	30	18.2	6.7-42.5

Determination of free myoinositol in amniotic fluid

Table IV gives the amounts of free myoinositol in amniotic fluid during the 2nd and 3rd trimester. The data do not show any clear difference between the two trimesters, possibly due to the large range observed. The collection of more than one sample per woman was not possible in this study.

Conclusion

The presence of pregnancy-dependent urinary oligosaccharides is confirmed in this study. The origin of the glycosides of myoinositol is still unclear, but their excretion follows the increased excretion of free myoinositol and it may therefore be assumed that free myoinositol is a requirement for their formation.

Serum (12,13), urine (13), and cerebrospinal fluid (14) of newborn infants have highly elevated amounts of free myoinositol. Adult levels are reached between the 2nd and 5th month post partum. Burton and Wells (15) demonstrated that in rats, the liver appears to be the main site of myoinositol biosynthesis during fetal life. The high content of myoinositol in the body fluids of the newborn infant can also in part depend on a dietary

supply, since human milk and particularly colostrum is a rich source of myoinositol (16).

In this study a high content of free myoinositol is found in amniotic fluid, the concentration being in the same order of magnitude as previously shown for cord blood (13). A considerable amount of free myoinositol was also found in the placenta, but we were unable to detect any of the myoinositol-containing oligosaccharides, and therefore the origin and function of these compounds remain obscure. It should also be pointed out that the physiological function of myoinositol itself, apart from its role in phosphoinositol biosynthesis, is not fully understood.

The high content of glycogen and glycogen degradation products, including $(\text{Glc})_4$, in placenta makes it highly possible that this organ is the main source of the increased urinary excretion of $(\text{Glc})_4$ during pregnancy.

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OLIGOSACCHARIDE EXCRETION IN URINE FROM HUMANS
AND THE RHESUS MONKEY

M. Alan Chester, Gudrun Lennartson, Bo S. Lindberg,
and Arne Lundblad

Department of Clinical Chemistry, University Hospital, Lund,
and Department of Obstetrics and Gynaecology,
Central Hospital, Västerås

Summary

A comparison has been made between the urinary neutral oligosaccharide excretion in humans and the Rhesus monkey. The overall excretion pattern was very similar, except for the presence of maltotriose as a major component of monkey urine. This trisaccharide is essentially absent from human urine. Unlike the blood group B-specific trisaccharide, maltotriose excretion was independent of food intake. These results indicate that the Rhesus monkey should be a useful animal model in studies on the mechanisms of oligosaccharide excretion.

Introduction

Human urine contains a number of oligosaccharides and glycopeptides which can show considerable variation in differ-

ent physiological and pathological conditions (1). The mechanisms by which these compounds are formed are mostly unknown. However, some information has been obtained about the formation of the dietary-dependent blood-group-active oligosaccharides using a Rhesus monkey as a suitable model (2). Using a gas-liquid chromatography-mass spectrometry method (3) we have compared the excretion of neutral oligosaccharides in normal human and Rhesus monkey urine, and have found sufficient similarities to justify the use of the Rhesus monkey as an experimental model for the study of the metabolism of these compounds.

Experimental Procedure

Materials. Urine was collected for the latter 12 h of a 24 h fasting period from two adult female Rhesus monkeys Macaca mulatta (both blood group B, secretors), and from a blood group B secretor woman during the last 4 h of a 16 h fasting period. Urine from non-starved monkeys and humans was collected without dietary restriction. Commercially available oligosaccharides, and oligosaccharides previously isolated from human urine were used as standards (Table I).

General Methods. All urines were frozen immediately after collection and kept at -20°C until required. The urines were processed by addition of 200 μg of isomaltotetraose as internal standard and then deionized by passage through columns (2 x 6 cm) packed with AG 50W-X8 (200-400 mesh) (H^{+}) and AG 3

TABLE I Structures of six neutral oligosaccharides from human urine

Trivial name	Structure	Ref.
xylosyl-glucose	xyl- α -(1-3)-glc	4
fucosyl-glucose	fuc- α -(1-2)-glc	4
fucosyl-inositol	fuc- α -(1- <u>O</u>)- <u>myo</u> inositol	5
glucose tetrasaccharide	glc- α -(1-6)-glc- α -(1-4)- -glc- α -(1-4)-glc	6
B-pentasaccharide	gal- α -(1-3)-gal- β -(1-4)-glc α -(1-2) α -(1-3) Fuc Fuc	7
B-trisaccharide	gal- α -(1-3)-gal α -(1-2) Fuc	8

All sugars are in the D-configuration except L-fucose.

(100-200 mesh) (OH^-) (Bio Rad Labs, Richmond, Calif.). The deionized urines were concentrated to dryness by rotatory evaporation ($<40^\circ\text{C}$), reduced and permethylated. Gas-liquid chromatography and mass spectrometry were carried out as described previously (3,9,10) and as indicated in the figures.

Results and Discussion

Reduction and methylation of deionised urine followed by

gas-liquid chromatography enables the neutral oligosaccharide pattern to be obtained. Due to the current limitations of the gas-liquid chromatographic procedure, the largest oligosaccharides detectable are pentasaccharides. The oligosaccharides of urine from fasting and non-fasting blood-group-B-secretor individuals are shown in Table II. The only qualitative difference observed is the presence in non-starved urine of the blood-group-B-trisaccharide (8). Examination of urine from two fasted Rhesus monkeys (blood-group-B-secretor) by gas-liquid chromatography-mass spectrometry gave a pattern similar but

TABLE II Neutral oligosaccharides found in normal human and Rhesus monkey urine

Compound	Human blood-group-B-secretor		Monkey blood-group-B-secretor	
	fasted	non-fasted	fasted	non-fasted
xylosyl-glucose	+	+	+	+
fucosyl-glucose	+	+	+	+
sucrose	+	+	+	+
lactose	+	+	+	+
maltose	+	+	+	+
isomaltose	+	+	+	+
fucosyl-inositol	+	+	+	+
hexosyl-N-acetyl-hexosamine	+	+	+	+
maltotriose	-	-	+	+
B-trisaccharide	-	+	-	+
glucose tetra-saccharide	+	+	+	+
B-pentasaccharide(s)	+	+	+	+

not identical to that obtained using fasted human urine (Fig. 1). The oligosaccharides detected are shown in Table II.

The dependence of the B-trisaccharide on diet was also observed in the Rhesus monkey (2). The identity of this compound was established by its identical retention time and mass spectrum when compared to an authentic sample (8).

The blood-group-B-pentasaccharide (7) was found in both human and monkey urine regardless of dietary status although the amounts in the monkeys were considerably less than in humans. A compound with a retention time slightly greater than the B-pentasaccharide (see Fig. 1, peak 12) was observed in

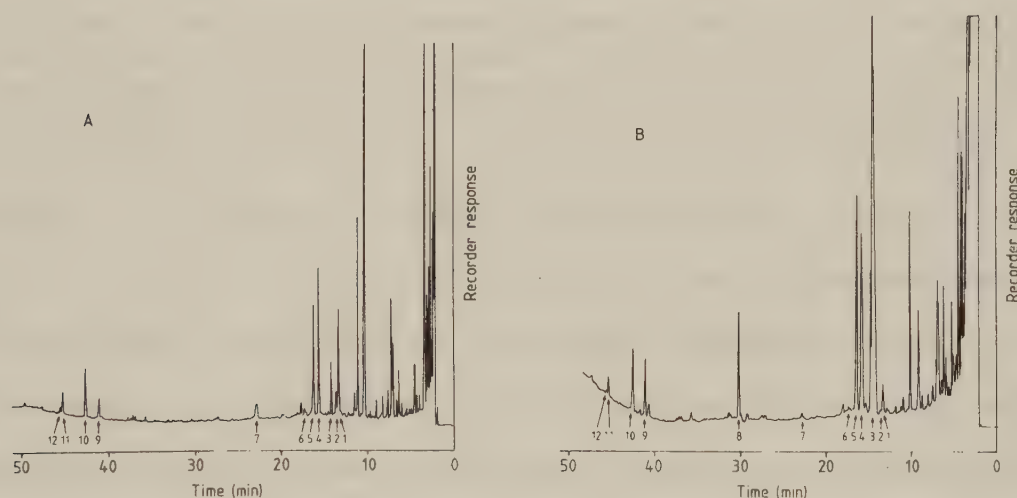


Fig. 1. Gas-liquid chromatogram of permethylated and reduced oligosaccharides from the urine of (A) a fasting blood-group-B-secretor individual and (B) a fasting blood-group-B-secretor Rhesus monkey. The separation was performed on a Perkin-Elmer 3920 gas chromatograph fitted with a glass capillary column, 0.25 mm x 25 m, wall-coated with SE-30 (LKB, Sweden) and a flame ionisation detector. Starting temperature, 150°C with a 4°C/min gradient up to 330°C.

Peak 1 = xylosyl-glucose; 2 = fucosyl-glucose; 3 = sucrose; 4 = maltose plus lactose; 5 = fucosyl-inositol; 6 = isomaltose; 7 = hexosyl-N-acetylhexosamine; 8 = maltotriose; 9 = glucose tetrasaccharide; 10 = isomaltotetraose (internal standard); 11,12 = B-pentasaccharides.

both human and monkey urine which had a mass spectrum compatible with that of the B-pentasaccharide, and was present only in blood-group-B individuals. These data indicate a structure similar to the B-pentasaccharide and may represent the equivalent compound with the type I backbone (i.e. Gal- α -(1-3)-[Fuc- α -(1-2)-]Gal- β -(1-3)-[Fuc- α -(1-4)-]Glc). The mass spectrum of these compounds would not be expected to differ significantly.

The glucose tetrasaccharide is excreted in low amounts (less than 2.5 mg/24 h) by normal individuals, but in greatly increased amounts in some types of glycogen storage disease and muscular dystrophy (11,12). The origin of this compound is not known, but it may arise from α -amylase action on glycogen (13). The two starved Rhesus monkeys excreted the glucose-tetrasaccharide within the normal human range, but if relative body weight is considered the monkeys excreted up to twice the normal maximum level.

Several disaccharides have been isolated from human urine, and characterized (4,5,14,15). Compounds having the same retention times and mass spectra can also be detected after gas-liquid chromatography-mass spectrometry of normal human urine. These compounds (xylosyl-glucose, fucosyl-glucose, fucosyl-inositol) are also present in Rhesus monkey urine. Fig. 2 shows a single ion trace of the xylosyl-glucose/fucosyl-glucose region of the gas-liquid chromatogram. Peak b in both human and monkey urine contains a permethylated deoxyhexose- unit (m/e 189) and a permethylated -(1-²H)hexitol unit (m/e 236). The retention time and mass spectrum are identical

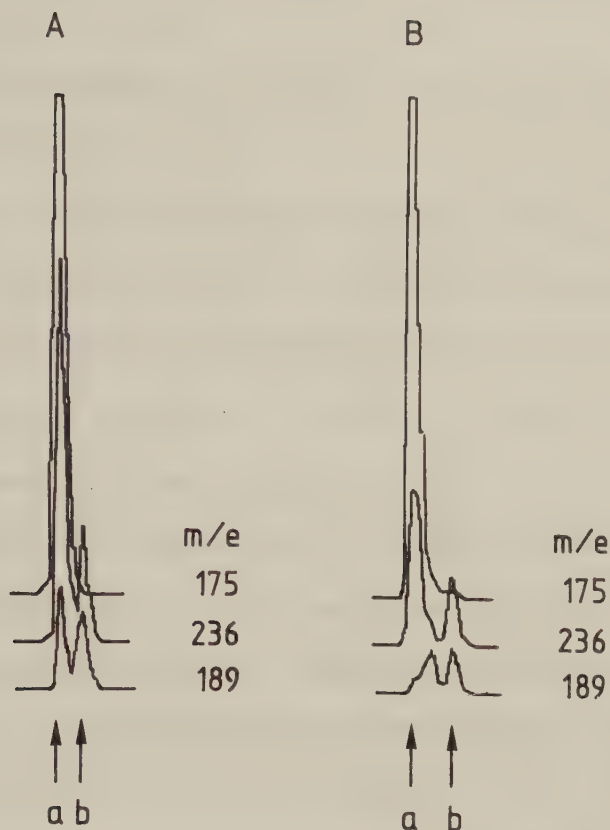


Fig. 2. Analysis of some disaccharides by mass spectrometry. The samples and gas-chromatographic procedure are as described in Fig. 1 except that the sugars were detected by single ion mass spectrometry. Only the xylosyl-glucose/fucosyl-glucose region is shown.

to those given by authentic permethylated and reduced (NaB^2H_4) fucosyl-glucose. Peak a contains a permethylated pentose-unit (m/e 175) and a permethylated and reduced $-(1\text{-}^2\text{H})$ hexitol unit (m/e 236) and has the same retention time and mass spectrum as permethylated and reduced (NaB^2H_4) xylosyl-glucose. This peak also contains a permethylated deoxyhexose-unit (m/e 189), and its mass spectrum, in addition to the expected pentose- $(1\text{-}^2\text{H})$ -hexitol fragments, also had fragments which indicated contamination with deoxyhexose- $(1\text{-}^2\text{H})$ hexitol. The presence of small amounts of another deoxyhexosyl (fucose?)-hexose in urine is indicated. Apart from fucosyl-glucose, the only other deoxyhexosyl-hexose known to occur in urine is fucosyl- α -(1-2)-

galactose, which, however, has a quite different retention time.

The presence of lactose, maltose, and isomaltose in human urine has been shown (14,15). In this study, both human and monkey urine gave peaks on gas-liquid chromatography with the same retention times as authentic lactose, maltose, and isomaltose. Their identities were confirmed by mass spectrometry. No gas-liquid chromatographic separation of lactose and maltose was achieved but these compounds were isolated from monkey urine by Sephadex G-25 and paper chromatography. Sucrose was detected in all urines but in considerably greater quantities in the monkey.

Normal individuals also usually excrete small quantities of a compound with a retention time between neutral di- and tri-saccharides. Its mass spectrum indicates a hexose-N-acetylhexosamine structure. This compound has not been isolated and characterized. It is also present in monkey urine.

The major qualitative difference between neutral oligosaccharides from human and monkey urine is the presence of a trisaccharide in the latter in appreciable amounts (5.6 mg compared to 0.3 mg glucose tetrasaccharide per 24 h fasted urine in one monkey, and 1.7 mg compared to 0.5 mg glucose tetrasaccharide per 24 h in the second monkey). This compound has an identical retention time on gas-liquid chromatography to maltotriose and its mass spectrum indicates the structure hexose-(1-4)-hexose-(1-4)-hexose (16). This compound could also be the product of α -amylase degradation of glycogen, the end products of which depend on the amount of enzyme and substrate available (13).

Of the oligosaccharides present in human urine, only the dietary dependent blood-group-B-specific trisaccharide has been studied in a way which demonstrated its origin (2,17). The Rhesus monkey appears to be a suitable model animal and should facilitate the study of oligosaccharide metabolism.

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